SURVIVAL, GROWTH AND THE POSSIBLE ENVIRONMENTAL IMPACTS OF INTRODUCED BLUE MUSSELS (*MYTILUS* SPP.) IN GEORGIA STRAIT, BRITISH COLUMBIA: IMPLICATIONS FOR MUSSEL AQUACULTURE.

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General Abstract

Marine invertebrate species have been introduced globally as a result of aquaculture and marine shipping. The primary purpose of this thesis was to examine the genetic and ecological implications of mussel introductions. Spatial and temporal comparisons of B.C. mussels made at large and small scales determined that introduced mussels are hybridizing with the native species (*Mytilus trossulus*). This hybrid zone is unstable and may remain so until environmental conditions favoring a successful invasion occur. Differences in survival and growth among mussel species and populations were examined to determine limits to commercial culture of mussels in B.C. The difference between native and introgressed mussel growth and survival rates was inconclusive. Local adaptation among Georgia Strait populations, however, was identified, representing a potential problem and important future consideration for aquaculturists. A technique was developed whereby mussels could be genotyped non-destructively, thus allowing pre-screening for larger sample sizes of non-native mussels. The results of this research have significant aquacultural and ecological implications and are discussed in terms of basic and applied science.

Table of Contents

List of Tables

List of Figures

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General Introduction

Mussels of the genus *Mytilus* are common inhabitants of the rocky intertidal and subtidal communities of the temperate zones of the northern and southern hemispheres (Suchanek 1985). Within the rocky shore community, the upper habitat limits of these mussels are generally the result of environmental pressures such as temperature and desiccation as well as competition with flora and fauna (Seed and Suchanek 1992). Lower zonational limits generally result from predation pressures especially from seastars, crabs and dogwhelks. Competition with other sessile invertebrates, including other mussel species (Harger 1970c), however, can also affect distribution, as can wave impact (Harger 1970a).

Most *Mytilus* populations reproduce seasonally via external fertilization, with spawning being triggered by environmental factors such as salinity and temperature (Lutz and Kennish 1992). Depending on conditions, spawning may be once or twice a year; however, gametes may be released throughout the year in areas with non-distinct seasonal changes (Lutz and Kennish 1992). Reproductive output varies according to body size and/or age with older/larger mussels allocating a greater proportion of available energy to reproduction and smaller mussels expending more for somatic growth (Bayne and Worrall 1980; Kautsky 1982). After fertilization, veliger larvae have an extended planktonic stage, typically one to four weeks depending on environmental conditions (Lutz and Kennish 1992). The combination of external fertilization and the extended pelagic stage of these mussels creates the potential for high dispersal capabilities. The larvae begin to metamorphosize, and are referred to as plantigrades just prior to settlement on a substrate, where metamorphosis to the bivalve form is completed (Lutz and Kennish 1992). The plantigrade mussels then grow into juveniles that will attach and detach themselves from unsuitable surfaces until a favorable habitat is encountered for the adult stage (Bayne

1976). These life history characteristics have resulted in the extensive geographical range of the *Mytilus* mussels as well as the high likelihood of contact between the species.

Within the genus *Mytilus*, the prevalence of interspecific hybridization as well as the morphological and ecological similarity of some of the species have resulted in taxonomic ambiguity. Species within the genus, however, are now being separated into different species based on molecular genetic data (McDonald and Koehn 1988; McDonald et al 1991; Gosling 1992b). The morphologically similar mussels which were once considered to be a single species ("*Mytilus edulis*") have been differentiated into the *Mytilus* sibling species complex which consists of three species: *M. edulis*, *M. trossulus* and *M. galloprovincialis* (McDonald et al. 1991). The differentiation of these species is supported by improved morphological analyses and molecular genetic data based on techniques such as the polymerase chain reaction (PCR) (Heath et al. 1995; Rawson et al. 1996).

The morphological similarity and extensive hybridization between members of this complex, have led to questions on their phylogenetic status. The classification of this group is critically dependent on the species definition that is used. The biological species concept distinguishes species based on reproductive isolation (Winston 1999). Based on this concept, the *Mytilus* sibling species would not be considered individual species, as members of the *Mytilus* sibling species complex have been found to hybridize and produce viable offspring in areas where their distributions overlap (Heath et al. 1995; Hilbish et al 2000). The phonetic species concept separates species based on morphological distinctiveness (Winston 1999). Again, it is difficult to define and separate the *Mytilus* species complex based on this concept due to their morphological similarity of shell shape, which is often influenced by environmental conditions (Inoue et al. 1997). The ecological species concept classifies groups by their occupation of different adaptive zones (Winston 1999). Ecologically, these mussels often overlap in their distributions, although this is considered to occur generally in areas of environmental intermediacy (Gosling 1992a).

Based on the criteria for each of these species concepts, it is difficult to classify the *Mytilus* sibling species as individual species. The phylogenetic species concept, however, can be used to differentiate the *Mytilus* sibling species. This concept defines a species as a single lineage descending from a common ancestor that is distinct from other lineages (Winston 1999). Recently, this species concept has been aided by molecular evidence. Molecular evidence, although still controversial, has demonstrated consistent differences amongst these three mussel species (McDonald and Koehn 1988; McDonald et al. 1991; Heath et al. 1995; Rawson et al. 1996). Physiologically, differences have also been noted within the *Mytilus* sibling species complex (Tedengren and Kautsky 1986; Hawkins and Bayne 1992). These differences, though, are less diagnostic due to their variability within the environment. Despite the controversy surrounding the taxonomic status of the *Mytilus* sibling species, it will be considered, for the purposes of this study, to consist of three distinct species: *M. edulis*, *M. trossulus* and *M. galloprovincialis*.

M. edulis is native to much of the Atlantic coast of the United States and Europe, but is also present in Australia, New Zealand and South America (Grant and Cherry 1985). It has also been introduced on the Pacific coast of North America for aquaculture purposes (Heath et al. 1995) (Figure i1). *M. trossulus* is native to parts of the eastern and western coasts of Canada as well as the Baltic Sea (Hilbish et al. 2000). *M. galloprovincialis* originated in the Mediterranean and the Atlantic coasts of southern Europe and North Africa (Gosling 1992a; Koehn 1991; McDonald et al. 1991; Suchanek et al. 1997; Hilbish et al. 2000). This species has since been introduced into several locations worldwide including England (Gardner 1994a), Japan (Wilkins et al. 1983), South Africa (Grant and Cherry 1985) and California (McDonald and Koehn 1988). These three mussel species also have been found to hybridize in areas where their distributions overlap (Figure i1). Hybrid zones have been found on the east coast of Canada between *M. edulis* and *M. trossulus* (Comesaña et al 1999; Penney and Hart 1999), on the southwest coast of England

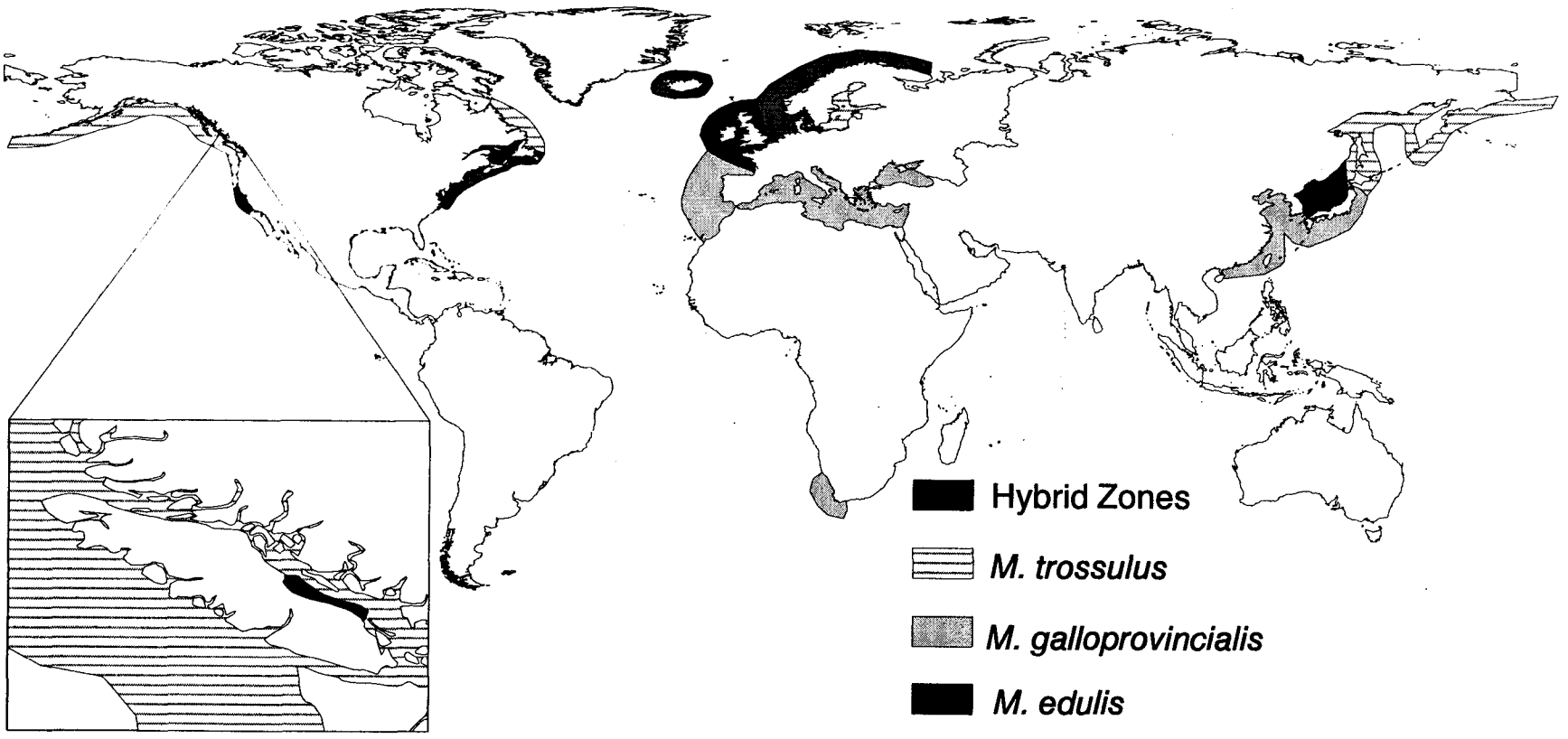


Figure i1. Approximate distribution of the *Mytilus* species complex showing areas of hybridization and sympatry between species pairs (Koehn 1991; McDonald et al. 1991; Heath et al 1995; Inoue et al 1997; Suchanek et al 1997; Wilhelm and Hilbish 1998; Comesaña et al 1999; Penny and Hart 1999; Rawson et al 1999; Hilbish et al 2000).

between *M. edulis* and *M. galloprovincialis* (Skibinski et al 1978; Gardner and Skibinski 1988; Gardner 1996; Wilhelm and Hilbish 1998), the north coast of Japan (Inoue et al. 1997), the North Pacific (California, Oregon, Washington) between *M. galloprovincialis* and *M. trossulus* (Suchanek et al. 1997; Rawson et al. 1999) and in Georgia Strait, BC between all three species of the *Mytilus* species complex (Heath et al. 1995) (Figure i1). For the context of this study, an introduction or introduced species is considered to be the result of human mediated transport (purposeful or otherwise). This distribution of the *Mytilus* species complex (Figure i1) is based on recent molecular data and historical results. Based on their disjunct distributions, it is likely, though, that some of these "native" distributions may themselves actually be introductions resulting from early shipping practices before the monitoring and recording of these organisms.

Mussels are an important component of the intertidal ecosystem with both ecological and socioeconomic value. Ecologically, mussels are keystone species in the rocky intertidal habitat, providing food, substrate and protection for other species. Mussels have been found to encompass up to 77% of the community dry weight and at an abundance of 700-4,000 individuals/m^2 in some areas (Hickman 1992). Due to this high biomass, a change or modification, even subtle differences, in this intertidal ecosystem through invasion and introgression of non-native mussels and alleles could have a large impact on the intertidal community by upsetting the organismal balance, and potentially affecting the entire food web.

Mussels also have a range of socioeconomic values from general and commercial food sources, to monitors of pollution and stress in ecosystems. In the past, mussels have been used as a food fishery by many coastal peoples and over the past century have increasingly been cultured for the commercial market. This commercial mussel culture has, in some cases, prompted the importation of non-native species to areas, and has generally been done without consideration of potential effects on the native marine communities. The

dominance of mussels in the intertidal community, and the fact that they are sedentary and suspension feeders, are some of the characteristics that have made mussels useful as monitors and bio-indicators, because contaminant concentrations in mussel tissue can be used to assess environmental conditions (Dame 1996). The importance of these marine invertebrates both ecologically and socioeconomically should therefore demonstrate the need for further clarification of the genus as well as consideration of environmental impacts by the commercial industry.

The primary purpose of this thesis was to examine the genetic and ecological implications of species introductions to British Columbia. The objectives of the experiments were to test for spatial and temporal variation as well as survival and growth differences of blue mussels (*Mytilus* species) in Georgia Strait, BC. The examination of spatial and temporal variation was conducted both on a large and a small scale to determine whether the distribution and abundance of the non-native mussels was expanding and whether the introgression of non-native alleles was spreading. Secondly, an examination of growth and survival differences between native and introgressed species and between different populations of native species was conducted to determine the commercial value of the native mussels (*M. trossulus*). The growth and survival of native and non-native, as well as local and transplanted mussels, were followed for one year to examine differences between the groups. Finally, a technique that will contribute to a better experimental design for growth and survival studies was developed; this work arose from the logistic problems associated with the growth and survival experiment. The use of a syringe to extract hemolymph permitted the genotyping of individual animals via DNA analysis without harming the animals.

Chapter 1: Invasion dynamics of blue mussels (*Mytilus* spp.) at large and small spatial and temporal scales in Georgia Strait, British Columbia.

1.1 Abstract:

The increasing rate of marine species introductions has highlighted concerns about invasion events in marine invertebrates. To examine the invasion dynamics of blue mussels in Georgia Strait at a large scale, mussels were collected in June from 1994 to 1998 at 10 sites along the east coast of Vancouver Island and the lower mainland of B.C. To examine the dynamics at a small scale, mussels were collected monthly from six sites around Chemainus, B.C. for a period of one year. These mussels were genotyped using three published diagnostic species markers: PLII, ITS and Glu-5'. Tests for spatial and temporal variation indicated that the native mussels (*M. trossulus*) are hybridizing with the introduced species (*M. edulis and M. galloprovincialis*). The resulting hybrid zone, however, does not appear to be stable temporally (changes noted in distribution and abundance among years), spatially (differences in distribution and abundance among sites) or genetically (unpredictable departures from Hardy-Weinberg Equilibrium (HWE) across space and time). The Georgia Strait hybrid zone did not follow any one of the existing hybrid zone models specifically. Instead it appears to be a combination of effects defined here as an "immigration mosaic hybrid zone." The patchiness of the sites pointed to a mosaic type of hybrid zone, which was supported by the variation in environmental conditions (salinity and temperature) among sites and over time. The instability of the zone, however, suggests that the introgressed mussels may not be well suited to the area and are dependent on sporadic introductions. The results of this study indicate that the introgression of alien alleles does not appear to be spreading through Georgia Strait at this time. Therefore these mussels may not currently be a significant threat to the native population or local flora and fauna. A significant change in environmental conditions (e.g.

global warming), however, could change this balance and provide the conditions necessary for an extensive invasion.

1.2 Introduction:

Over the past century, increased sea traffic and commercial culture of aquatic organisms have led to an increase in the rate of change in the distribution and abundance of marine organisms. Species have been redistributed through accidental transfer via transport and discharge of ballast water, fouling and boring organisms on wooden ships and on exploratory drilling platforms, as well as through intentional introductions of non-native or alien species for commercial purposes (Carlton 1989; Carlton and Geller 1993; Ruiz et al. 1997; Carlton 2000).

Intentional introduction of marine organisms continues to increase as a response to over-fishing of wild stocks, disease and environmental degradation (Lipton et al 1992). Unfortunately, the potential impacts of intentional introductions on local species and communities are rarely considered. The impact of these introductions, however, can range from little or no negative impact to extensive problems and changes in local communities. Potential negative impacts include the importation of "piggyback" organisms (disease microogranisms, parasites, predators, competitors) along with the target species (Carlton 1992; Carriker 1992), as well as genetic changes to the local communities through hybridization with the imported species (Carlton 1992; Carriker 1992; Gaffney and Allen 1992). As a result, it is imperative that risk assessments be conducted before the importation of non-native/alien species to an area.

The introduction of molluscs, and bivalves in particular, has received widespread attention due to the extensive transport of these organisms around the world for commercial purposes (Chew 1990; Carlton 1992; Carriker 1992; Gaffney and Allen 1992; Lipton et al. 1992). Oysters, for example, have long been commercially harvested and, as a result of

over-harvesting wild stocks and the desire to create new fisheries, many species have been transferred around the world. Generally, these transfers have been done with little consideration of consequences, and as a result, introductions of unwanted species have occurred resulting in problems with commercial fisheries as well as non-commercial species (Chew 1990; Carlton 1992; Carriker 1992; Lipton et al. 1992; Robinson 1999). Over the last few years commercial rearing and harvesting of imported blue mussel species on the Pacific coast has increased, and again there has been little consideration of the potential environmental impact of this practice. Heath et al. (1995) examined the distribution of introduced alleles from *M. galloprovincialis and M. edulis* using two species marker loci and discussed the introgression of these alleles into the native species, *M. trossulus* within Georgia Strait. These introduced alleles were determined to have introgressed into the native *M. trossulus* population differently depending on locus and site within Georgia Strait, with the highest levels of introgression around the south east coast of Vancouver Island. The impact of this introduction, however, was undetermined.

Springer and Heath (2002) continued sampling in 1995 on the south-east coast of Vancouver Island, where the highest incidence of non-native ("alien") alleles was thought to be located based on the results of Heath et al (1995). Shell length of all mussels sampled was measured and preliminary results suggested that a size differential might exist between the native and introgressed blue mussels, with the introgressed mussels attaining larger sizes. This suggestion is consistent with the results of Gardner and Skibinski (1988) and Wilhelm and Hilbish (1998) which showed that the *M. galloprovincialis* genotypes were generally larger than *M. edulis* genotypes. Comesaña et al. (1999) and Penney and Hart (1999) also showed that the *M. edulis* genotypes were generally larger than *M. trossulus* genotypes. The potential impact that this size differential may have on the environment includes the ability of predators to feed on the mussels, potentially affecting the balance of the intertidal community. It has been shown that some mussel predators such as seastars

(*Pisaster*) and eider ducks (*Somateria mollissima*) are unable to feed on mussels beyond a certain size class (Paine 1976; Bustnes and Erikstad 1990; Bustnes 1998; Hamilton et al 1999). The selection of smaller mussels (natives) by these predators could slowly create a population of larger sized mussels (introgressed), if predation is primarily of adults. This may or may not upset the balance of the intertidal community.

The introduction of a species can be complicated by the potential for hybridization with native species and the introgression of alien alleles into the native population. Natural hybridization occurs when genetic incompatibilities between the two taxa or populations are not too great (Harrison 1993; Gardner 1996; Arnold 1992). Successful hybridization results in fertile F1 hybrids and backcrosses (hybrid crossed with a pure-type) (Harrison 1993; Gardner 1996; Arnold 1992). Interspecific hybridization has been found to exist in many marine invertebrates such as sea urchins (Lessios and Pearse 1996), oysters (Allen and Gaffney 1993), crabs (Bert and Arnold 1995), clams (Dillon and Manzi 1989), and mussels (Coustau et al. 1991; Gardner 1995; Saavedra et al. 1996). Hybrid zones occur when genetically distinct groups of organisms meet and mate, resulting in at least a portion of viable offspring of mixed ancestry (Harrison, 1993). Generally hybrid zones are maintained by a balance between selection against unfit hybrid genotypes and dispersal capabilities ("tension zone" hypothesis), but pose a threat to genetic diversity only if the hybrids are viable (Harrison 1993). Hybrid zones can eventually result in the fusion of distinct species into a single species, the replacement of one hybridizing parental species by another, or the strengthening of reproductive barriers through selection against hybrids (Harrison 1993; Arnold 1992). Hybridization, however, can also have a positive effect on species/ populations by genetically enriching rarer forms resulting in elevated fitness and broader environmental tolerances (Arnold 1992).

There are 3 primary models that have been proposed to explain the maintenance of hybrid zones. They differ in the importance of environmental effects and in the fitness of

hybrids. The *ecotonal model* put forward by Endler (1997) suggests that hybrid zones are maintained by hybrid adaptation to environmental conditions intermediate to those of bordering parental conditions. The *tension model* developed by Barton and Hewitt (1989) suggests that hybrid zones are not the result of environmental conditions or by hybrid viability, but of consistent dispersal into the area by parental genotypes. Finally, the *mosaic model* (Harrison and Rand 1989) suggests that parents are adapted to discrete habitat patches and where patches overlap, hybrids form as a result of environmental heterogeneity.

Extensive hybridization among members of the *Mytilus* species complex has led to some debate over the genus' taxonomic status; however, recent molecular genetic analysis has shown evidence of significant genetic differentiation (McDonald and Koehn 1988; McDonald et al. 1991; Heath et al. 1995; Rawson et al. 1996). *Mytilus* species hybridize in several locations throughout their range (Skibinski et al. 1978; Gardner and Skibinski 1988; Viard et al. 1994; Gardner 1994a and b; Gardner 1996; Suchanek et al. 1997; Comesana et al. 1999; Figure i1). Some hybrids among members of the *Mytilus* sibling species complex have been found to be viable and in some cases have demonstrated increased fitness (Beaumont et al. 1993), although reduced fitness is generally characteristic of hybrids within stable hybrid zones (Skibinski et al 1978). Various studies have been conducted to examine the distribution and extent of hybridization of the *Mytilus* species on the Pacific coast of North America. The majority of those studies have concentrated on *Mytilus galloprovincialis* and *Mytilus trossulus* within the California hybrid zone (McDonald and Koehn 1988; Shaw et al. 1988; Geller et al. 1994; Rawson and Hilbish 1995; Rawson et al. 1999). Georgia Strait, which lies between Vancouver Island and the southern coast of British Columbia, Canada, is another area where hybridization between *M. trossulus* and two introduced mussel species, *M. galloprovincialis* and *M. edulis*, has been documented (Heath et al 1995; Springer and Heath 2002).

When examining genetic variation in biological systems it is necessary to take into consideration the scale over which the study system is assessed. Spatial variation, or geographic structuring, is important to consider when studying the population dynamics of any species that is not endemic to a single habitat. Spatial scale is important as the degree of genetic variation or structure of a species or population may increase or decrease depending on the scale at which it is examined (Gosling 1992b). The degree of population structuring within a species will vary depending on life-history characteristics and ecological requirements of the species as well as degree of habitat fragmentation, natural or otherwise (Scribner et al. 1997). Increasing habitat fragmentation may create structural barriers, and potentially separate neighboring populations from one another. Variables such as population size, selection, and the rate of migration and gene flow are also factors that can affect genetic differentiation among sub-populations (Bowen 1982). Geographic variation is often studied at two scales: macrogeographic variation (variation over a large distance) and microgeographic variation (variation over a small distance) (Gosling 1992b). Clearly, the scale of geographic variation will depend on the dispersal capabilities of the organism.

Theoretically, mussels are not expected to have a high degree of microgeographic genetic structuring due to their extended pelagic larval stage that can potentially disperse the larvae great distances (Lutz and Kennish 1992). The larvae can also be transported in ballast water, and juveniles moved on the hulls of ships, thereby greatly increasing the dispersal capabilities and distribution of these mussels over space and time (Carlton and Geller 1993). The pelagic larval stage, as well as human mediated transportation, should result in extensive dispersal and mixing of different populations and species, thus eliminating physical mating barriers to make these species genetically homogeneous (Gosling 1992b). Numerous mussel populations and species, though, have been found to maintain genetic structure resulting in complex and patchy distribution patterns both on the macro- and microgeographic scales (Suchanek et al. 1997). Genetic structuring has been

found to be the result of environmental factors such as temperature (Suchanek et al. 1997), salinity (Sarver and Foltz 1993), wave exposure (Harger 1970a; Boulding et al 1999), and oceanographic currents (Sanjuan et al. 1996) as well as viability differences offset by replacement through immigration (Wilhelm and Hilbish 1998). It has thus been proposed by Gardner (1996) that genetic structure in mussels is maintained, despite dispersal capabilities, hybridization and introgression, by environmental adaptation.

Given the morphological and ecological similarity of the *Mytilus* species complex, the question arises as to whether it matters if there is one species or three species (or hybrids or backcrosses) on the coast of British Columbia and why this system should be studied. Ecologically, problems could arise if the introgressed mussels had a negative effect on the environment. Changes in the distribution and abundance of mussel populations could also indicate changes in environmental conditions (Inoue et al. 1997). In evolutionary terms, the study of species complexes and hybrid zones aids in the understanding of speciation through the examination of gene flow between populations and species, natural selective forces among populations and species, the amount of genetic differentiation involved, and the scale over which it occurs (Skibinski et al. 1978; Viard et al. 1994).

The purpose of this study was to examine spatial and temporal changes in introgressed allele frequencies in Georgia Strait, B.C. Part of the study was conducted on both temporally and spatially large scales – 10 sites over approximately 350 km of coastline over 5 years (1994 to 1998). Mussels were collected at each site each year and were genotyped to determine the patterns of distribution and abundance of the *Mytilus* sibling species, their hybrids and backcrosses. The null hypothesis was that there would be no change in the distribution or abundance of non-native alleles over space and time (i.e., a stable hybrid zone). The second part of the study included sampling on a microgeographic scale around Chemainus, B.C. within a short temporal scale. Mussels were sampled

monthly for one year from each of six sites, all within approximately 20 km of each other, and genotyped to determine the pattern of distribution and abundance of non-native alleles over a smaller geographic area and shorter time intervals. The null hypothesis was that there would be no differences in distribution or abundance of introgressed alleles at these scales (i.e., short-term hybrid zone stability).

1.3. Materials and Methods:

1.3.1. Field Sampling – Large Scale:

The sampling for this study was designed to examine the progression of an alien allele invasion over time and space as well as the stability of a new hybrid zone. The introduced, non-native ("alien") alleles were both *M. edulis* and *M. galloprovincialis.* Any mussel genotyped having an alien allele at any one of the three marker loci was deemed an "introgressed" mussel. Only those individuals which were genotyped as having the native (*M. trossulus*) alleles at all three loci were identified as "native".

In 1994 twelve sites along the east and west coasts of Vancouver Island and the lower mainland of British Columbia were sampled to study mussel invasions on the west coast of Canada (Heath et al. 1995). In 1995, mussels were collected from three of the original 12 sites: Chemainus, Nanaimo, and French Creek (Figure 1.1). In 1996, 1997 and 1998, 10 sites within Georgia Strait (along the east coast of Vancouver Island and the lower mainland) were sampled. Sites were chosen within Georgia Strait to match those of Heath et al. (1995) so that the new data could be compared to the existing data.

In June of each sampling year, mussels were collected subtidally from private or Federal Government docks at each site. Docks were chosen for easy access and because they represented similar microenvironments at each site. A collection of mussels was retrieved from each dock and from this, a random sub-sample of 30-50 mussels was

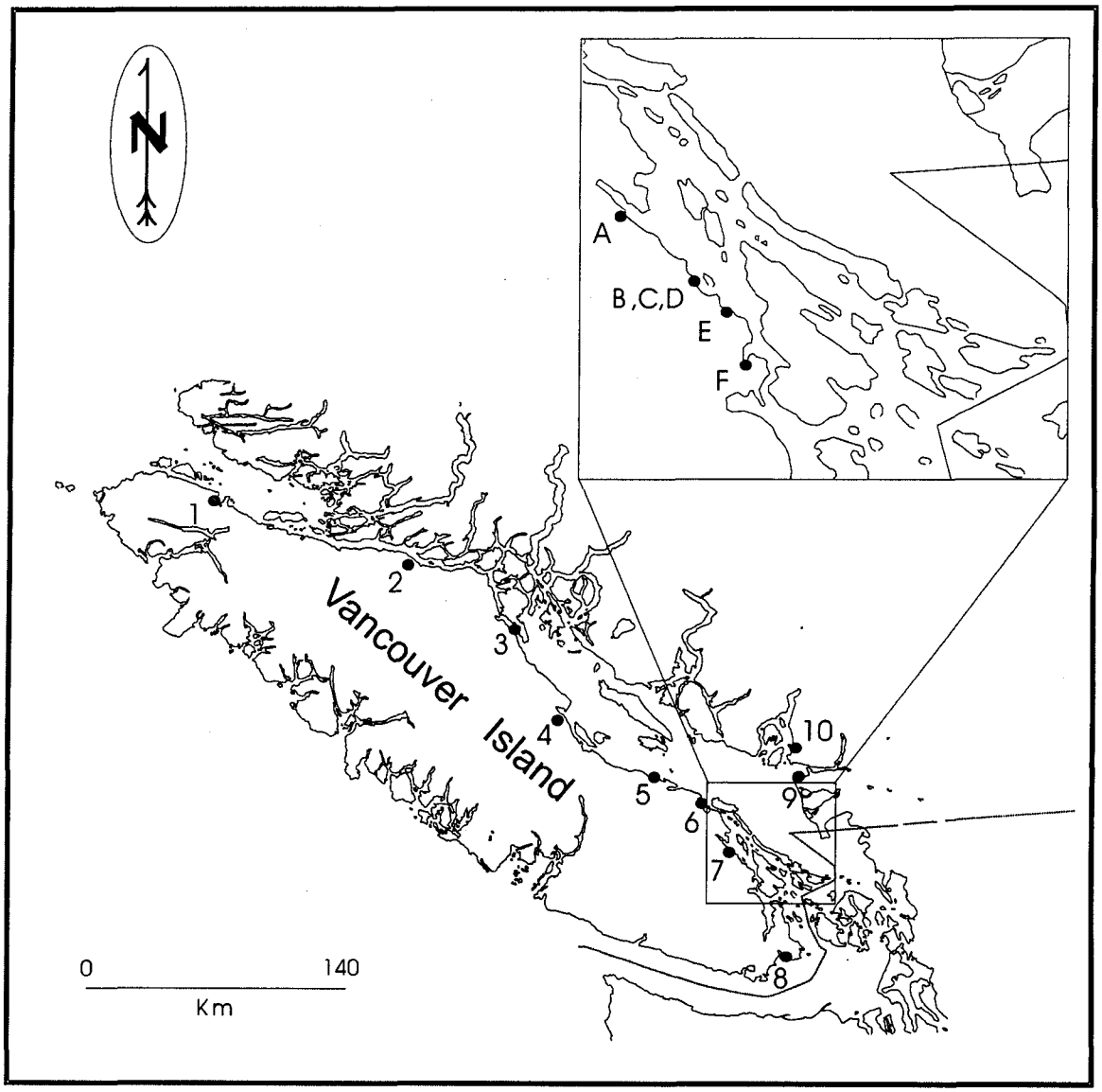


Figure 1.1 – Map of sampling sites (both large and small scale) within Georgia Strait, B.C.. Site 1 is Port Hardy, 2 is Sayward, 3 is Yellow Island Aquaculture Ltd. (Quadra Island), 4 is Union Bay, 5 is French Creek, 6 is Nanaimo, 7 is Chemainus, 8 is Moses Point, 9 is South Vancouver (Kitsilano) and 10 is West Vancouver (Horseshoe Bay). A is Ladysmith, B, C and D are the three sites within Chemainus, E is Crofton and F is Maple Bay.

selected. A minimum of thirty mussels was collected as required to detect the presence of alien alleles at any one site based on recommendations by Heath et al. (1995). Tissue samples from the mantle edge or margin were taken and preserved in 95% ethanol for individuals over 10 mm in shell length. Smaller mussels (<10 mm) were preserved whole in ethanol. Shells from all samples were measured (± 0.01 mm) with digital calipers to determine total length (from the tip of the beak to the posterior edge).

1.3.2. Field Sampling - Small Scale:

Sampling at a smaller geographic scale was centered on the region of historically high introgression. The data from 1994 and 1995 (Heath et al 1995, Springer and Heath 2001), indicated temporal and small-scale variation in introgression, but the scale was geographically too large and the time intervals too far apart to detect specific changes. The small scale sampling was designed to provide data over a short time interval and small geographic scale to document changes in the area suspected of being critical in the invasion of Georgia Strait by alien mussels and the introgression of alien alleles into the native mussel population.

Six sites within and surrounding Chemainus, B.C. were selected for intensive sampling. Three of the sites were located within Chemainus (Figure 1.1 inset). One site was on the outer side of the government wharf and the second was on the inside of the government wharf (approximately 30 m between the two sites). These two sites were similar in that both had a high degree of commercial and recreational boat traffic; however, the outer side had frequent large boat traffic and appeared to have more water flow, whereas the inner side had small boat traffic and appeared to have less water flow. The third site in Chemainus was a commercial dock, approximately 100 m north of the government wharf (Figure 1.1 inset), which had good water flow and large boat traffic. The remaining three sites were located outside of Chemainus. One was at the government

dock in Crofton, B.C. (approximately 10 km south of Chemainus), which had both pleasure boat and ferry traffic, the second was at the government dock in Maple Bay, B.C. (approximately 15 km south of Chemainus) which was in a sheltered bay with little boat traffic (Figure 1.1 inset). The final site was at the government dock in Ladysmith, B.C., approximately 10 km north of Chemainus (Figure 1.1 inset). This dock had both commercial and pleasure boat traffic, and was adjacent to a sawmill. Sites were sampled monthly from April 1998 to May 1999 to examine changes in distribution and abundance of *Mytilus* (natives and introgressed). Each collection consisted of 50 mussels from each site (except for Ladysmith in January, 1999 as there were insufficient numbers to obtain a full sample). The same protocol as used in the large scale was used to collect and prepare the samples. A water sample was taken at each site on each sampling date to measure salinity and a temperature data logger (Onset Computer Corp., Pacasset, MA) was placed at each site to record temperature. Salinity and water temperature measurements were taken in the small-scale study to determine environmental differences that could impact blue mussel population dynamics since these two parameters are most critical to mussel survival and growth. Mean daily water temperatures were calculated and compared among the six sites. Salinity measurements were also compared for all sites.

1.3.3. DNA Extraction and Genotype Analysis – Large and Small Scale:

DNA Extraction:

For each mussel, approximately 20-100 mg of mantle margin tissue was removed from the ethanol, in which it had been preserved, and dried by pressing the ethanol out onto a sterile tissue. In cases where the mussel was too small to obtain a sample of mantle tissue, either half or the whole mussel was used. The dried tissue (or mussel) was digested in 200 µL of lysis buffer (50 mM Tris-HCL (pH 8.0); 1.0% SDS; 25 mM ethylenediaminetetraacetic acid (EDTA)) with 200 µg proteinase K overnight at 37°C. After

digestion, samples with shell remnants were centrifuged at 13 000 rpm for 5 minutes to separate the shell fragments from solution. The supernatant was removed and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1), followed by isopropanol precipitation and 70% ethanol wash (Heath et al., 1995). The extracted DNA was resuspended in 100 μL of nanopure water along with 1 μL of RNAse, which was added to degrade RNA in the sample. Random samples of DNA were chosen to run on a 1.8% agarose gel to verify quality and quantity of DNA present.

Genotype Analysis/Nuclear DNA markers:

Mussels were identified as introgressed (*M. edulis*, or *M. galloprovincialis*, hybrid, backcross), or native (*M. trossulus*) using three molecular genetic markers. The markers based on the internal transcribed spacer (ITS) region, and the protamine-like sperm packaging protein coding region (PLII) were first described in Heath et al (1995) (Table 1.1). Restriction fragment length polymorphism (RFLP) analysis is used with both of these markers since the use of an appropriate restriction enzyme on the PCR product will produce diagnostic banding patterns, which can be used to genotype individuals. The ITS marker is a codominant marker that differentiates between *M. trossulus*, alien or hybrid genotypes (but not specific species of alien). PLII is a dominant marker that differentiates only native or alien genotypes; hybrids are identified as natives. The third marker, which targets the gene coding for the polyphenolic adhesive protein (Glu-5'), was first described in Rawson et al. (1996). This marker (long form) is different than the first two in that restriction enzymes are not required to genotype individuals. Instead, there is diagnostic variation in the fragment lengths of the amplified PCR product (PCR-FLV) which can be used to genotype individuals (Table 1.1). The Glu marker is a codominant marker which is able to differentiate between *M. trossulus, M. edulis, M. galloprovincialis* as well as their hybrids. A modified form (short form) of the Glu-5' marker was developed for this study to enable

multiple, simultaneous sample analysis, with the long and short form of the marker on an auto-sequencer (Table 1.1). Each of these three markers are diagnostic species markers which enabled the analysis of spatial abundance and distribution of these mussels, as well as hybrids and backcrosses.

Table 1.1. Locus name, primer sequences, marker type, associated restriction enzyme, and resultant diagnostic fragment size in base pairs (bp) for the *Mytilus* species complex (Heath et al. 1996; Rawson et al. 1996). Note: PCR-FLV refers to PCR-fragment length variation.

Locus	Primer Sequence	Marker Type	Restriction Enzyme	Fragment Size (bp)		
				Native	Alien	
				MT	MG	ME
ITS	5'-GTTTCCGTAGGTGAACCTG-3'	RFLP	*Hha* I	180	180	180
	5'-CTCGTCTGATCTGAGGTCG-3'			280	450	450
PLII	5'-GAGCCCAAGTAGGAAATCCCG-3'	RFLP	*Hinf* I	100	100	100
	5'-CCTTCGCATTGTTACATTTATT-3'			200		
Glu-5' (long)	5'-GTAGGAACAAAGCATGAACCA-3'	PCR-FLV	_	229	189	244
	5'-GGGGGGATAAGTTTTCTTACC-3'					
Glu-5' (short)	5'-GTAGGAACAAAGCATGAACCA-3'	PCR-FLV	_	136	91	145
	5'-GGGGGGATAAGTTTTCTTAGG-3'					

ITS:

Polymerase chain reaction (PCR) amplifications consisted of 1 µL of mussel DNA, 0.2 mM of each of the four deoxynribonucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 50 ng of the appropriate primers (forward and reverse), 1.0 unit of recombinant Taq DNA polymerase (Gibco-BRL), PCR buffer and nanopure water to a total volume of 15µl. The thermal cycler protocol consisted of an initial denaturation temperature of 94°C for 60 s, followed by 35 cycles of: a denaturation temperature of 94°C for 60 s, an annealing temperature of 52°C for 60 s, and an extension temperature of 72°C for 60 s followed by a final extension temperature of 72°C for 5 minutes. Each PCR reaction was checked for successful amplification by running the product on a 1.8% agarose gel at 100 volts for 20 minutes. A sample from the amplified PCR product was then digested with restriction

endonuclease *Hha* I. For each endonuclease digestion reaction, 5 - 10 μL (depending on strength of PCR) of each amplified PCR-product was digested for 4 hours at 37°C with 0.5 units of *Hha* I, 1.5 μL of buffer supplied by the manufacturer (Gibco-BRL) and brought to a total of 15 μL with nanopure water. The digested products were electrophoresed for 2 hours at 60 volts on 2.5% agarose gels stained with ethidium bromide (0.4% EtBr (0.5μg/ml)). The restriction fragment length polymorphisms (RFLPs) were visualized by ultraviolet transillumination of the gels. Individual mussels were scored for genotype at the ITS locus on the basis of diagnostic RFLPs (Heath et al. 1995; see Table 1.1).

PLII:

A protocol similar to that for the ITS locus was used for the PLII locus. The same PCR conditions were used except for a lower annealing temperature (50 °C). The PCR fragments were digested as above, with *Hinf* I as the restriction enzyme, and the gels were transilluminated with ultraviolet light to score for PLII genotype (Heath et al. 1995; see Table 1.1).

Glu-5':

An automated DNA sequencer was used to score genotypes at the Glu-5' locus, since some of the diagnostic bands were differentiated by only a few base pairs, making it difficult to reliably score on an agarose gel. Polyacrylamide gels allowed reliable determination of *Mytilus trossulus*, *M. edulis*, *M. galloprovincialis* and hybrid genotypes.

PCR amplifications consisted of 15-μL reaction mixtures containing 1 μL of mussel DNA, 0.2 mM of each of the four deoxynribonucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 0.1 mM of the dye-labeled forward Glu-5 (Table 1.1) and 0.2 mM of the reverse (short or long form) Glu-5' (Gibco-BRL), 0.6 unit of Recombinant Taq DNA polymerase (Gibco), PCR buffer and nanopure water. The thermal cycler protocol consisted of an initial

denaturation at 94°C for 60 s, followed by 30 cycles of a denaturation temperature of 94°C for 30 s, an annealing temperature of 55°C for 30 s, an extension temperature of 72°C for 30 s and a final extension temperature of 72°C for 5 minutes.

The amplified PCR products were then scored for genotype using an automated DNA sequencer (Visible Genetics, Toronto, ON) and known size standards (100 and 200 bp) to determine amplified fragment length and the resultant genotype identification. Each run consisted of two sets of 15 samples (one sample set was amplified with the short Glu-5' primer, one set with the long Glu-5' primer) along with one set of known species controls (*M. trossulus*, *M. edulis*, *M. galloprovincialis*) for comparison. The size standards were used to differentiate between the two runs and enabled the identification of species bands. Up to three runs were possible on one gel. Thus, 2 sample sets x 2 dyes x 3 runs = potential for 12 genotypes / lane. Multiple uses of the acrylamide gel allowed for rapid genotyping of a large number of individuals at a reasonable cost. The sensitivity of the sequencer allowed for the genotyping of individuals with very little sample DNA and/or weak PCRs and also enabled the identification of *M. edulis* versus *M. galloprovincialis*.

1.3.4. Data Analysis

The same population genetic analysis was utilized at both large and small scales. Unless otherwise stated, all genetic analyses were performed using *Tools for Population Genetic Analyses* (TFPGA 1.3) software developed by Mark Miller (Biology Dept.; Arizona State University, PO Box 871501, Tempe, AZ 85287-1501, USA).

An exact test for Hardy-Weinberg Equilibrium (HWE) (no allele class pooling) was employed at each locus, within each group (site/population by year for the large scale and by month for the small scale). The Monte Carlo method included a total of 1000 dememorization steps; 10 batches; 2000 permutations/batch (Raymond and Rousset 1995). The resulting estimates of the probability of departure from HWE were corrected

using the sequential Bonferroni correction (Rice 1989) to account for multiple simultaneous tests (36 tests for the large scale and 38 tests for the small scale).

Native and introgressed allele frequencies at each site in each year or each month were determined. An exact test for allele frequency distribution differences was used to test for changes in introgression levels both among sample years/months (within populations) and among populations (1000 dememorization steps; 10 batches; 2000 permutations/batch; Raymond and Rousset 1995) at each locus. Post-hoc pairwise comparisons, using the exact test, were used to examine differences between specific populations at specific times, and were also subject to the sequential Bonferroni correction. Post-hoc tests were only used on adjacent sites as comparisons between distant sites were not considered meaningful.

Shell length comparisons were made between introgressed and native individuals. Shell lengths were compared between the two groups for mussels from Chemainus only from 1994 to 1998. The 1994 mussels were included for historical reference, although Heath et al.'s (1995) use of only two loci for genotyping may have misidentified a small number of native individuals. Chemainus was chosen for the size comparison since that site had data for all time periods sampled, and generally had the highest level of introgressed individuals. Shell length comparisons of native and introgressed mussels were also made using data from the small scale experiment using mussels from Ladysmith for all time periods. Ladysmith was chosen for the size comparisons since that site had data for all time periods sampled.

1.4. Results

Sample Collections:

For the large scale study, Heath et al.'s study (1995) provided historical data for all sites except South Vancouver (Table 1.2). In 1995, mussels were only collected from

Table 1.2. Temporal and spatial proportion of introgressed alleles in Georgia Strait, B.C. between 1994 and 1998. Top number is the percent (%) of introgressed alleles, value in parentheses is number of individuals genotyped. "-" designates no samples collected.

	ITS					PLII					GLU				
	1994	1995	1996	1997	1998	1994	1995	1996	1997	1998	1994	1995	1996	1997	1998
Port Hardy	1.7 (29)	_	0.0 (35)	1.0 (48)	0.0 (47)	0.0 (39)	_	0.0 (18)	0.0 (41)	2.0 (50)	_	_	0.0 (40)	1.0 (48)	2.0 (50)
Sayward	0.0 (35)	_	0.0 (29)	0.0 (45)	0.0 (42)	5.1 (39)	_	7.1 (28)	0.0 (40)	6.7 (45)	_	_	0.0 (29)	0.0 (41)	2.0 (50)
YIAL	0.0 (40)	_	0.0 (48)	0.0 (40)	1.1 (44)	6.8 (44)	_	0.0 (35)	0.0 (34)	2.2 (45)	_	_	0.0 (47)	0.0 (37)	0.0 (47)
Union Bay	3.9 (26)	_	0.0 (40)	1.8 (28)	2.1 (48)	0.0 (30)	_	3.5 (29)	0.0 (37)	12.8 (47)	_	_	0.0 (42)	1.2 (43)	1.0 (50)
French Creek	7.1 (35)	1.7 (120)	0.0 (41)	0.0 (45)	0.0 (48)	4.5 (22)	6.9 (29)	0.0 (32)	13.3 (30)	15.2 (33)	_	_	0.0 (45)	0.0 (36)	0.0 (47)
Nanaimo	5.2 (29)	2.6 (133)	0.0 (43)	3.8 (40)	0.0 (50)	3.3 (30)	1.7 (60)	2.6 (39)	9.7 (31)	0.0 (45)	_	_	0.0 (46)	3.2 (32)	0.0 (49)
Chemainus	13.8 (29)	32.7 (124)	2.1 (47)	1.4 (110)	2.5 (242)	6.9 (29)	27.4 (124)	0.0 (46)	0.0 (53)	8.4 (261)	_	33.5 (124)	2.2 (46)	1.7 (176)	1.5 (261)
Moses Point	0.0 (30)	_	1.1 (47)	8.5 (47)	0.0 (47)	3.3 (30)	_	3.0 (33)	6.5 (46)	4.7 (43)	_	_	1.0 (49)	9.4 (48)	1.3 (38)
South Van	_	_	0.0 (39)	0.0 (33)	0.0 (41)	_	_	0.0 (33)	0.0 (22)	5.0 (40)	_	_	0.0 (44)	1.1 (44)	0.0 (48)
West Van	0.0 (35)	_	0.0 (42)	0.0 (35)	1.3 (38)	0.0 (26)	_	7.1 (28)	0.0 (8)	7.9 (38)	_	_	1.1 (47)	0.0 (17)	0.0 (47)

Chemainus, French Creek and Nanaimo. In 1996, 1997 and 1998, mussels were collected from all 10 sites (Table 1.2). For the small-scale study, mussels were collected from all 6 sites from April, 1998 to July, 1998 (Table 1.3). In August, 1998, mussels were only found and collected at the Chemainus commercial dock and at Ladysmith. From October, 1998 until the end of the sampling period, mussels were only found and collected at the Ladysmith site, although all sites were visited and checked for mussels (Table 1.3).

Hardy-Weinberg Equilibrium:

Along the east coast of Vancouver Island and mainland Vancouver, most of the sampled sites were in HWE over time (Table 1.4). Significant departures from HWE (after Bonferonni correction), occurred in 1995 at French Creek and Nanaimo, in 1997 at Moses Point and in 1994, 1995 and 1998 at Chemainus. There were also marginally significant deviations at these sites as well as at Union Bay in 1994 and 1998 (Table 1.4). Marginally significant results have been defined as results that are statistically significant before Bonferonni correction, but are rendered non-significant after Bonferonni correction (Ferguson 1996). Port Hardy, YIAL, Union Bay, French Creek, Nanaimo, West Vancouver, Sayward and South Vancouver had no detectable alien alleles for certain years and therefore HWE could not be tested (Table 1.4).

In the small scale study, there were no significant deviations from HWE (after Bonferonni correction) at Crofton and Maple Bay, during the time period that mussels were present (April to July, 1998) (Table 1.5). There was one sample date when Maple Bay deviated marginally significantly from HWE (June, 1998). Mussels were not found at Crofton or Maple Bay during the remaining sampling dates (August, 1998 to May, 1999). Within Chemainus, the inner and outer government dock sites were out of HWE in April, 1998, and had no alien alleles to test from May to July, 1998 (Table 1.4). Neither site had mussels for the remainder of the sampling dates (August, 1998 to May, 1999). The mussel

Table 1.3. Temporal and microgeographic spatial proportion of introgressed alleles in the area surrounding Chemainus, B.C. between April, 1998 and May, 1999. Top number is the % of introgressed alleles, value in parentheses is the number of individuals genotyped. 'Cr' is Crofton, B.C., 'MB' is Maple Bay, B.C., 'La' is Ladysmith, B.C., 'Ch-O' is the outer side of the government wharf in Chemainus, B.C., 'Ch-I' is the inner side of the government wharf in Chemainus, B.C., and 'Ch-C' is a commercial dock to the left of the ferry terminal in Chemainus, B.C. "-" designates no samples available for collection.

	ITS						PLII						GLU					
	Cr	MB	La	Ch O	Ch I	Ch C	Cr	MB	La	Ch O	Ch I	Ch C	Cr	MB	La	Ch O	Ch I	Ch C
4/98	0.0 (50)	0.0 (48)	9.4 (32)	4.1 (49)	4.1 (49)	1.0 (50)	0.0 (49)	8.1 (37)	5.1 (39)	5.1 (39)	5.1 (39)	1.3 (38)	0.0 (49)	1.1 (45)	6.4 (39)	2.2 (46)	0.0 (43)	2.5 (40)
5/98	0.0 (49)	1.0 (50)	1.0 (48)	0.0 (50)	0.0 (50)	4.0 (50)	2.0 (49)	8.7 (46)	2.1 (48)	2.2 (45)	0.0 (44)	4.6 (44)	0.0 (43)	0.0 (50)	1.2 (43)	0.0 (50)	0.0 (49)	3.0 (50)
6/98	3.1 (49)	2.0 (50)	4.2 (48)	0.0 (50)	0.0 (49)	0.0 (50)	14.9 (47)	6.1 (49)	2.4 (41)	0.0 (43)	0.0 (31)	2.1 (47)	3.0 (50)	0.0 (50)	4.0 (50)	2.0 (50)	0.0 (39)	0.0 (50)
7/98	4.0 (50)	1.0 (50)	12.8 (47)	0.0 (49)	0.0 (50)	0.0 (50)	4.2 (48)	4.0 (50)	8.5 (47)	2.0 (49)	0.0 (46)	2.1 (48)	4.1 (49)	1.0 (50)	10.6 (47)	0.0 (49)	1.0 (50)	0.0 (50)
8/98	_	_	28.1 (48)	_	_	3.0 (50)	_	_	18.9 (37)	_	_	8.3 (36)	_	_	25 (48)	_	_	0.0 (49)
10/98	_	_	40 (45)	_	_	_	_	_	31.6 (38)	_	_	_	_	_	17.1 (38)	_	_	_
1/99	_	_	85 (10)	_	_	_	_	_	69.2 (13)	_	_	_	_	_	75 (18)	_	_	_
4/99	_	_	25.9 (27)	_	_	_	_	_	26.9 (26)	_	_	_	_	_	14.9 (47)	_	_	_
5/99	_	_	5.6 (45)	_	_	_	_	_	14.3 (35)	_	_	_	_	_	2.9 (35)	_	_	_

Table 1.4.a. Exact tests for Hardy-Weinberg Equilibrium of alien alleles in Georgia Strait, B.C. between 1994 and 1998. H.O. is the observed number of heterozygote alleles, H.E. is the expected number of heterozygote alleles. p-value is statistically significant at 0.05. p-values with an asterisk '*' denote significant departure from HWE after Bonferonni correction.

Site	Year	ITS			GLU		
		H.O.	H.E.	p	H.O.	H.E.	p
Port Hardy	1994	1	1	1.00	0	0	-
	1996	0	0	-	0	0	-
	1997	1	1	1.00	1	1	1.00
	1998	0	0	-	2	2	1.00
Sayward	1994	0	0	-	0	0	-
	1996	0	0	-	0	0	-
	1997	0	0	-	0	0	-
	1998	0	0	-	2	2	1.00
YIAL	1994	0	0	-	0	0	-
	1996	0	0	-	0	0	-
	1997	0	0	-	0	0	-
	1998	1	1	1.00	0	0	-
Union Bay	1994	0	2	0.020	0	0	-
	1996	0	0	-	0	0	-
	1997	1	1	1.00	1	1	1.00
	1998	0	2	0.0090	1	1	1.00
French Creek	1994	1	5	0.0025	0	0	-
	1995	2	6	0.0007*	0	0	-
	1996	0	0	-	0	0	-
	1997	0	0	-	0	0	-
	1998	0	0	-	0	0	-
Nanaimo	1994	1	3	0.052	0	0	-
	1995	3	7	0.0012*	0	0	-
	1996	0	0	-	0	0	-
	1997	1	3	0.036	2	2	1.00
	1998	0	0	-	0	0	-
Chemainus	1994	0	7	0.0000*	0	0	-
	1995	15	55	0.0000*	25	55	0.0000*
	1996	2	2	1.00	2	2	1.00
	1997	1	3	0.013	6	6	1.00
	1998	2	12	0.0000*	8	8	1.00

Table 1.4.b. Continuation of Table 1.4.a.

Site	Year	ITS			GLU		
		H.O.	H.E.	p	H.O.	H.E.	p
Moses Point	1994	1	1	1.00	0	0	-
	1996	1	1	1.00	1	1	1.00
	1997	2	7	0.0002*	3	8	0.0019
	1998	0	0	-	1	1	1.00
South Van.	1996	0	0	-	1	1	1.00
	1997	0	0	-	1	1	1.00
	1998	0	0	-	0	0	-
West Van.	1994	0	0	-	0	0	-
	1996	0	0	-	1	1	1.00
	1997	0	0	-	0	0	-
	1998	1	1	1.00	0	0	-

population at the nearby Chemainus commercial dock was significantly out of HWE in May, 1998 after Bonferonni correction; and marginally significant deviation from HWE in August, 1998 (Table 1.5). Ladysmith was the only site to maintain a mussel population throughout the sampling period (April, 1998 to May, 1999). This site was out of HWE in April, July, August and October, 1998, and April and May, 1999 (Table 1.5). In all the above cases, those sites out of HWE were the result of too few hybrids being found.

Table 1.5. Results from exact test for Hardy-Weinberg Equilibrium of alien alleles in the six sites around Chemainus, B.C. between April, 1998 and May, 1999. H.O. is the observed number of heterzygote alleles, H.E. is the expected number of heterozygote alleles. p-value is statistically significant at 0.05. p-values with an asterisk '*' denote statistically significant departures from HWE after Bonferonni correction.

Site	Month	ITS			GLU		
		H.O.	H.E.	p	H.O.	H.E	P
Ladysmith	4/98	0	5	0.0000*	1	5	0.0030
	5/98	1	1	1.00	1	1	1.00
	6/98	2	4	0.059	4	4	1.00
	7/98	0	10	0.0000*	0	9	0.0000*
	8/98	5	19	0.0000*	12	18	0.046
	10/98	2	22	0.0000*	11	11	1.00
	1/99	1	3	0.16	7	7	1.00
	4/99	1	10	0.0000*	4	12	0.0000*
	5/99	1	5	0.0015*	0	2	0.015
CH-Com. Dock	4/98	1	1	1.00	2	2	1.00
	5/98	0	4	0.0006*	1	3	0.030
	6/98	0	0	-	0	0	-
	7/98	0	0	-	0	0	-
	8/98	1	3	0.033	0	0	-
CH-Inner Dock	4/98	0	4	0.0001*	1	1	1.00
	5/98	0	0	-	0	0	-
	6/98	0	0	-	0	0	-
	7/98	0	0	-	1	1	1.00
CH-Outer Dock	4/98	0	4	0.0002*	0	2	0.0097
	5/98	0	0	-	0	0	-
	6/98	0	0	-	0	2	0.0093
	7/98	0	0	-	0	0	-
Crofton	4/98	0	0	-	0	0	-
	5/98	0	0	-	0	0	-
	6/98	3	3	1.00	3	3	1.00
	7/98	2	4	0.062	4	4	1.00
Maple Bay	4/98	0	0	-	1	1	1.00
	5/98	1	1	1.00	0	0	-
	6/98	0	2	0.011	0	0	-
	7/98	1	1	1.00	1	1	1.00

Geographical Comparison: Large Scale Study

There was considerable variation in introgressed allele frequency at all three loci in space and time, mainly centered around SE Vancouver Island (Table 1.2, Figure 1.2). Frequency of introgressed individuals also fluctuated with a crash in numbers noted in 1996 followed by an increase at most sites in 1997 and 1998 (Appendix I).

Based on the sites sampled, there was an overall significant difference in levels of introgressed alleles among the sample sites in 1994 (ITS), 1995 (all 3 loci), 1996 (ITS and Glu) and 1997 (all 3 loci). There were no significant differences in introgression levels overall or among the sites in 1998 due to few or no introgressed alleles (Table 1.6, Figures 1.2, Appendix 1). Using post-hoc pairwise comparisons, it was also determined that no significant differences occurred between adjacent sites after Bonferonni correction in 1994 or 1996. In 1995, Chemainus had significantly higher introgression levels than Nanaimo ($p \leq 0.0001$ at all three loci), but no difference was noted between Nanaimo and French Creek (PLII: p=0.25, ITS and Glu: p=1.00) (Figures 1.2, Appendix I). In 1997, no significant differences in introgression levels were found among the sites at the PLII locus, but at the ITS and Glu loci, Moses Point was found to have a significantly higher level of introgressed alleles than Chemainus ($p \leq 0.0001$) (Figure 1.2).

Table 1.6. Combined exact test results for spatial differences in levels of introgression in Georgia Strait, B.C.

	Combined Exact Test Probability Results						
	PLII		ITS		Glu		ITS + Glu
Year	S.E.	p	S.E.	p	S.E.	p	p
1994	0.018	0.25	0.0004	0.0004	N/A	N/A	N/A
1995	0.000	0.000	0.000	0.0000	N/A	N/A	N/A
1996	0.0096	0.26	0.0012	0.0039	0.0020	0.0064	0.0003
1997	0.0011	0.0012	0.0006	0.0009	0.0022	0.0045	0.0001
1998	0.012	0.087	0.020	0.29	0.0098	0.63	0.50

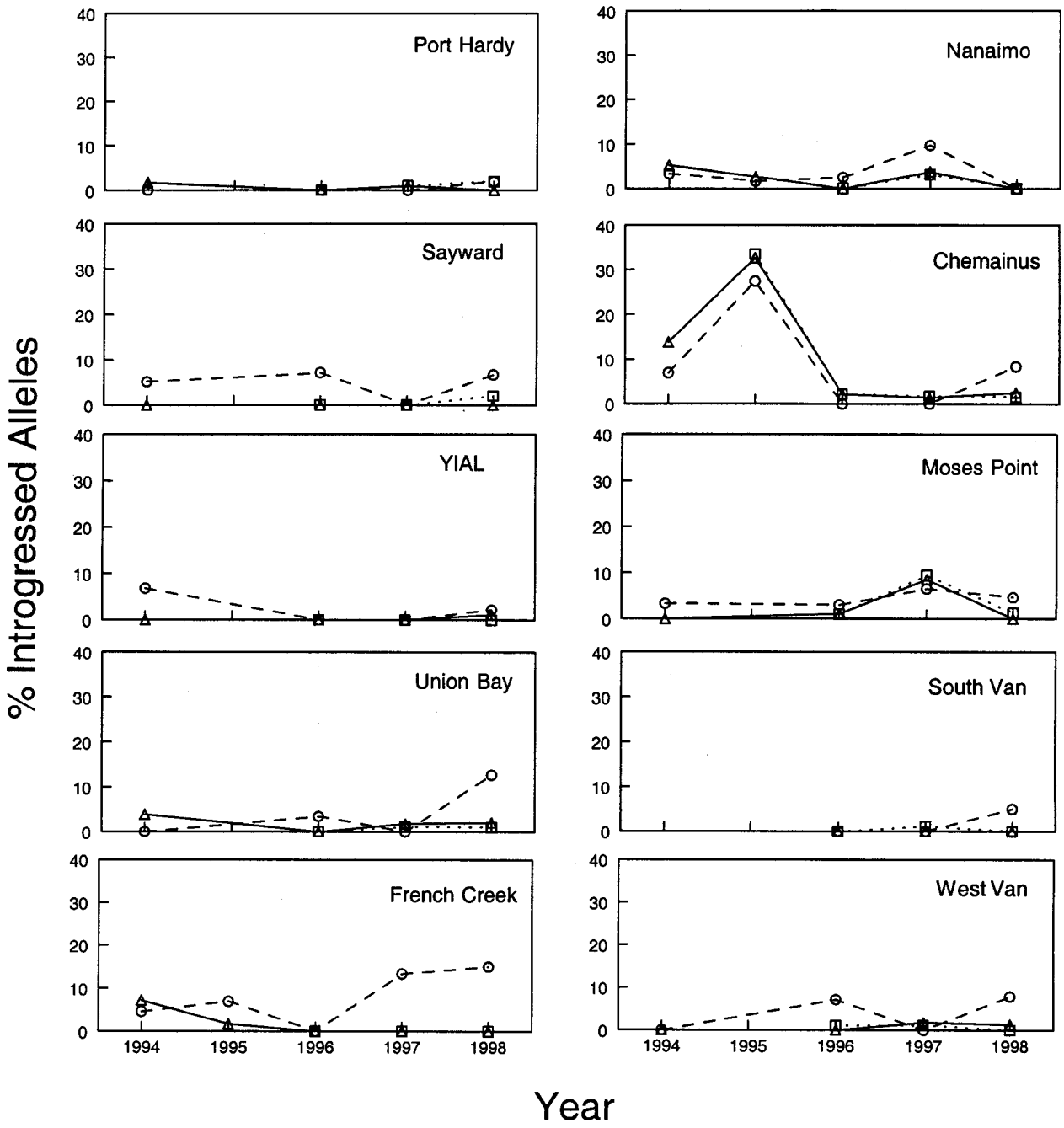


Figure 1.2. Proportion of introgressed alleles as a function of time in Georgia Strait, B.C. from the north end of Vancouver Island (Port Hardy) to the South (Moses Point) and the mainland (South Van and West Van) between 1994 and 1998. ▲ - denotes proportion genotyped at the ITS locus, O - denotes proportion genotyped at the PLII locus, □ - denotes proportion genotyped at the GLU locus.

Geographical Comparison: Small Scale

There was considerable variation in introgressed allele frequency at all three loci in space and time (Table 1.3). Comparisons among sites were only made until August 1998 since after this time, only Ladysmith maintained a population of mussels (Table 1.3). At the PLII locus, an overall significant difference in introgression levels was only noted in June 1998 (Table 1.7). Post-hoc pairwise comparisons of adjacent sites, however, found no significant differences after Bonferonni correction.

At the ITS and Glu loci, overall significant differences in introgression levels were noted among sites on all dates analysed (Table 1.7). Generally, the highest proportion of introgressed alleles and individuals was at the Ladysmith site for all time periods except June 1998 when Crofton showed a slightly higher incidence than Ladysmith (Figures 1.3 and Appenidix II). In April, May and June 1998, post-hoc pairwise comparisons found no difference between sites after Bonferonni correction (Figures 1.3, Appendix II). In July 1998, post-hoc pairwise comparisons found a significantly greater level of introgression in Ladysmith than each of the Chemainus sites ($p \leq 0.0001$ for each test), but no difference among the other sites (Figures 1.3, Appendix II). The significant difference in overall introgression levels in August 1998 (Table 1.7) was due to the higher level of introgression in Ladysmith compared to the Chemainus commercial dock as these were the only two sites to have mussel populations on this date (Figures 1.3, Appendix II). Spatial comparisons could not be made beyond August 1998 due to the lack of mussel populations at any site except Ladysmith (Figures 1.3, Appendix II).

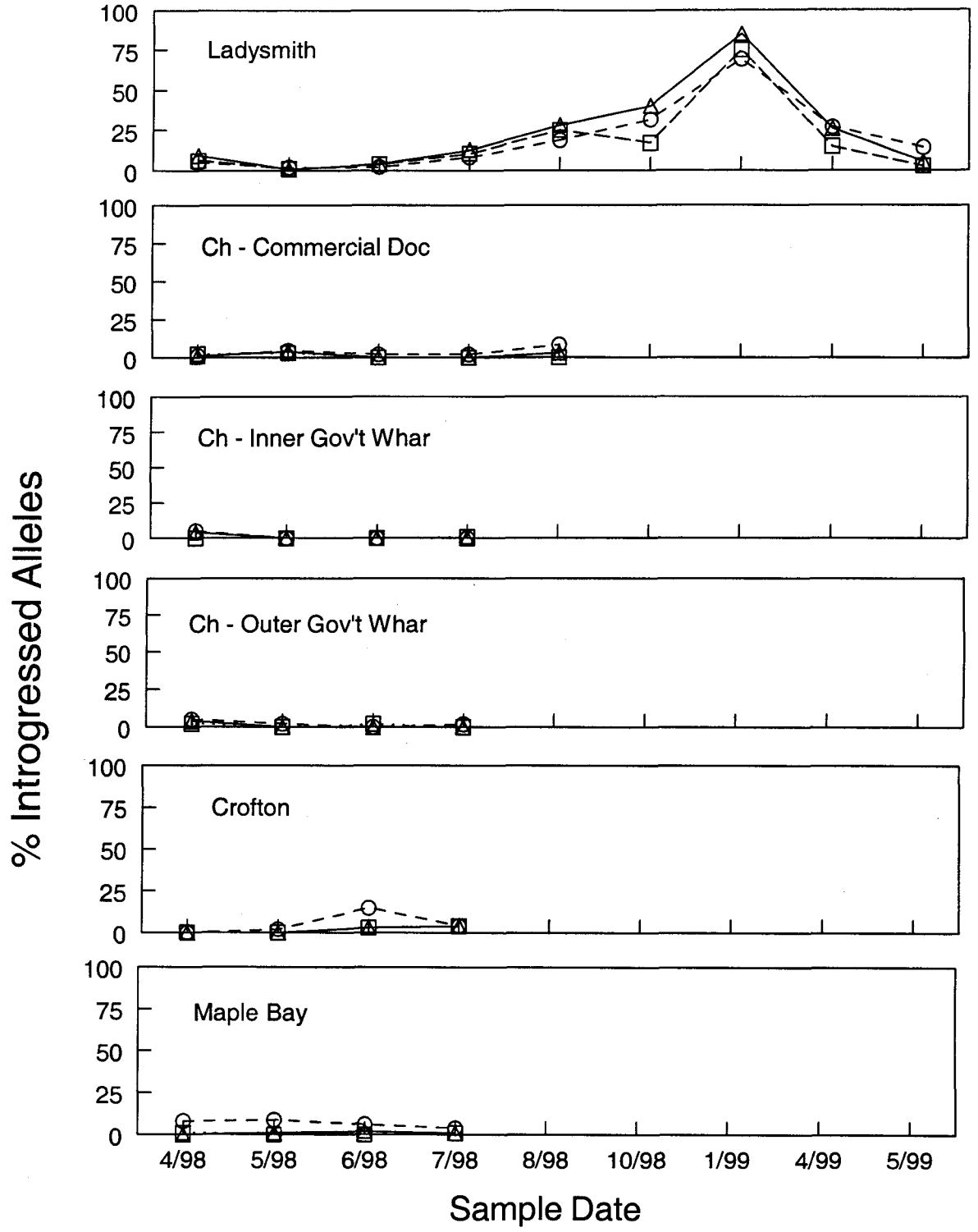


Figure 1.3. Proportion of introgressed alleles as a function of time at six different sites (between April 1998 and May 1999) surrounding the suspected area of highest alien allele incidence, Chemainus, B.C. Δ - denotes proportion genotyped at the ITS locus, O - denotes proportion genotyped at the PLII locus, □ - denotes proportion genotyped at the GLU locus.

Table 1.7. Combined exact test results for spatial differences in levels of introgression at the six sites selected around Chemainus, B.C.

	Combined Exact Test Probability Results						
1998	PLII		ITS		Glu		ITS + Glu
Month	S.E.	p	S.E.	p	S.E.	p	P
April	0.013	0.44	0.0006	0.0012	0.0077	0.095	0.0011
May	0.0091	0.33	0.0050	0.046	0.0040	0.056	0.018
June	0.0048	0.035	0.0053	0.021	0.0046	0.058	0.0092
July	0.012	0.35	0.0000	0.0000	0.0000	0.0000	0.0000
August	0.0071	0.32	0.0000	0.0000	0.0000	0.0000	0.0000

Temporal Comparison: Large Scale

Significant temporal changes in introgression levels at the PLII locus were only found in Union Bay and Chemainus (Table 1.8), although post-hoc comparisons of subsequent years in Union Bay were not found to be significantly different after Bonferonni correction (Figures 1.2, Appendix I). Significant temporal changes in introgression levels at the ITS/GLU loci were found in French Creek, Nanaimo, Chemainus and Moses Point. Post-hoc comparisons of successive years found no significant differences in introgression levels in French Creek and Nanaimo after Bonferonni correction (Figures 1.2, Appendix I).

In Chemainus, based on PLII results, the level of introgression only changed significantly from 1995 to 1996 (after Bonferonni correction) when there was a significant decrease ($p \leq 0.0000$) in introgression levels (Figures 1.2, Appendix I). There was no change in introgression levels after this point. At the ITS locus, there was a significant increase in introgression levels (after Bonferonni correction) from 1994 to 1995 ($p=0.0048$) followed by a significant decrease in introgression levels (based on the ITS and Glu loci) from 1995 to 1996 ($p \leq 0.0000$) (Figures 1.2, Appendix I). No significant change in introgression levels occurred after this point (Figures 1.2, Appendix I).

In Moses Point, post-hoc comparison between years determined that the level of introgression increased significantly ($p=0.0014$) from 1996 to 1997, followed by a significant

decrease (p=0.0025) in levels form 1997 to 1998 at the ITS/GLU loci (Figures 1.2, Appendix I).

Table 1.8. Combined exact test results for temporal differences in levels of introgression within Georgia Strait, B.C. from the most northern point to the most southern. 'PH' is Port Hardy, 'Sa' is Sayward, ' YIAL' is Quadra Island, 'UB' is Union Bay, 'FC' is French Creek, 'Na' is Nanaimo, 'Ch' is Chemainus, 'MP' is Moses Point, 'SV' is South Vancouver (Kitsilano), and 'WV' is West Vancouver (Horseshoe Bay). "*" denote a significant difference after Bonferonni correction.

	Combined Exact Test Probability Results						
	PLII		ITS		Glu		ITS + Glu
Site	S.E.	p	S.E.	P	S.E.	p	p
PH	0.0000	1.00	0.0088	0.54	0.0050	0.78	0.7876
Sa	0.0098	0.32	0.000	1.00	0.0069	0.51	0.8558
YIAL	0.0050	0.29	0.0062	0.72	0.0000	1.00	0.9554
UB	0.0040	0.024	0.0098	0.30	0.0000	1.00	0.6616
FC	0.0086	0.19	0.0014	0.0063	0.0000	1.00	0.0380
NA	0.11	0.15	0.0066	0.051	0.0032	0.067	0.0228
Ch	0.0000	0.0000*	0.0000	0.0000	0.0000	0.0000*	0.0000*
MP	0.0065	0.63	0.0007	0.0021	0.0018	0.0078	0.0002*
SV	0.0044	0.52	0.0000	1.00	0.0045	0.54	0.8710
WV	0.0069	0.59	0.0071	0.71	0.0000	1.00	0.9518

Temporal Comparison: Small Scale

Overall, temporally significant changes in introgression level were found only in Ladysmith and Crofton, B.C. at the PLII locus (Table 1.9). After post-hoc pairwise comparisons, however, no significant differences in introgression levels were found between sampling dates at either site (Figures 1.3, Appendix II). At the ITS and GLU loci, overall changes in introgression levels were noted at all sites except for Maple Bay (Table 1.9). Post-hoc comparisons of successive years, however, only showed significant changes in Ladysmith (Figures 1.3, Appendix II). After Bonferonni correction, a significant increase in introgression levels was detected between July 1998 and August 1998 followed by no change between August 1998 and October 1998. Another significant increase ($p \leq 0.0000$) in introgression levels occurred between October 1998 and January 1999, followed

by a significant drop in introgression levels ($p \leq 0.0000$) from January, 1999 to April, 1999 and again from April 1999 to May 1999 ($p=0.0003$) (Figures 1.3, Appendix II).

Table 1.9. Combined exact test results for temporal differences in levels of introgression at the six sites selected around Chemainus, B.C. CH-C is the Chemainus commercial dock, CH-I is the inner side of the Chemainus government dock and CH-O is the outer side of the Chemainus government dock. "*" denote a significant difference after Bonferonni correction.

	Combined Exact Test Probability Results						
	PLII		ITS		Glu		ITS+Glu
Site	S.E.	p	S.E.	p	S.E.	p	P
Ladysmith	0.0000	0.0000*	0.0000	0.0000	0.0000	0.0000	0.0000*
CH-C	0.011	0.66	0.0047	0.066	0.0042	0.053	0.023
CH-I	0.011	0.18	0.0024	0.010	0.0045	0.86	0.050
CH-O	0.0072	0.50	0.0021	0.0077	0.0064	0.23	0.015
Crofton	0.0015	0.0049	0.0051	0.040	0.0056	0.058	0.016
Maple Bay	0.0066	0.80	0.0032	0.90	0.0053	0.61	0.88

Size Comparisons: Large Scale

Size comparisons of introgressed and native mussels were made over time using mussels sampled from Chemainus. In 1994, the introgressed mussels were significantly larger than the native mussels ($p \leq 0.001$). In 1995, the introgressed mussels were again significantly larger than the native mussels ($p \leq 0.001$), although overall, the mean size had not changed from the previous year (Figure 1.4). The mean size of the introgressed mussels dropped between 1995 and 1996 resulting in no significant size differences ($p=0.210$) between the introgressed and native mussels as the native mussels had remained approximately the same size (Figure 1.4). Size differences also were not significant from 1996 to 1997 ($p=0.247$). Differences in size became significant again in 1998 ($p \leq 0.001$) as the introgressed mussels became larger again (Figure 1.4). Large variation in the standard errors of the introgressed mussels was due to low sample numbers (n_{94}= 4 of 29, n_{95}= 63 of 124, n_{96}= 2 of 44, n_{97}= 6 of 54, n_{98}= 19 of 157).

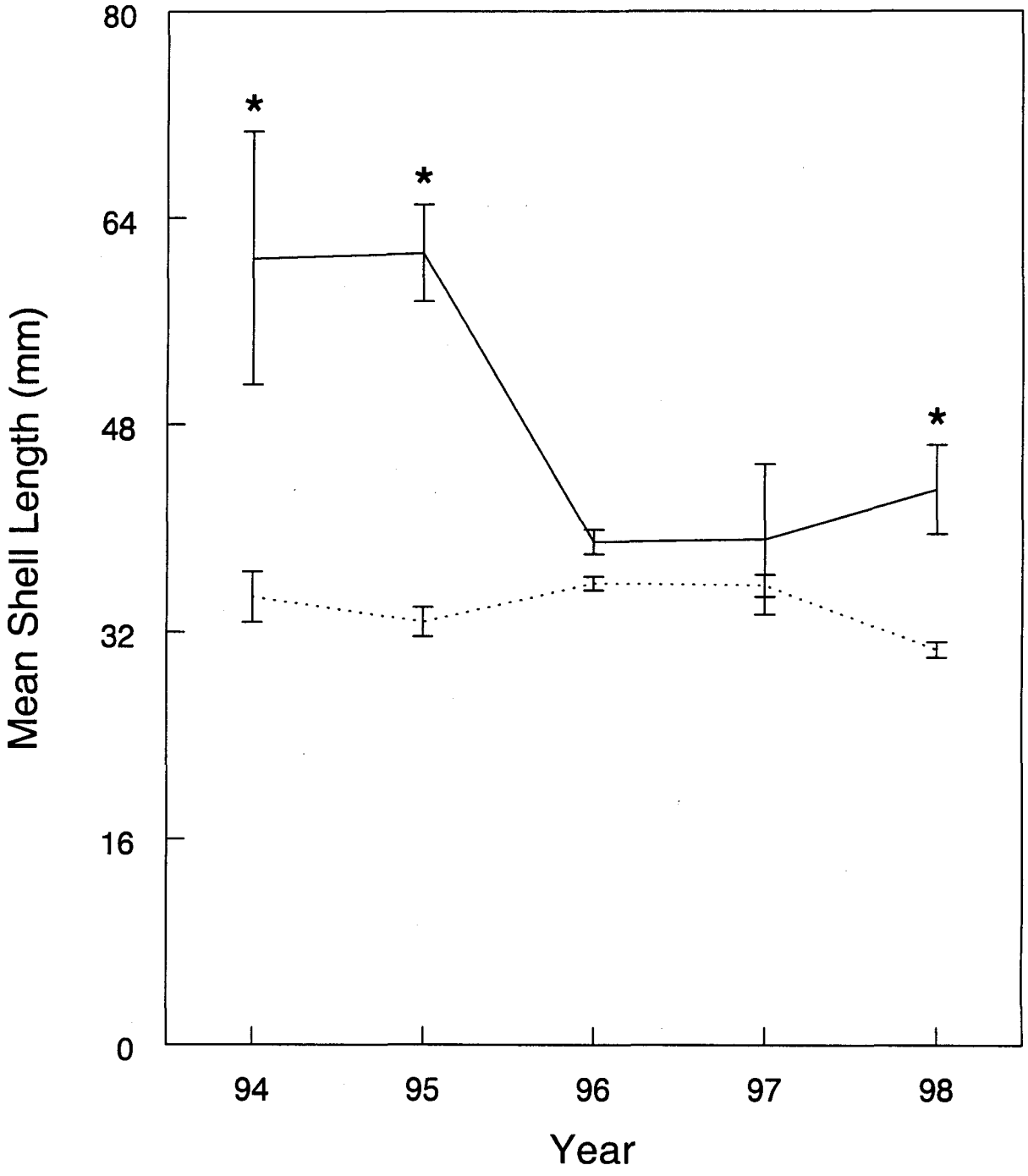


Figure 1.4. Temporal comparisons of mean shell length differences between introgressed (solid line) and native (dotted line) mussels from Chemainus, B.C. (large scale sampling) between 1994 and 1998.

Size Comparisons: Small Scale

Mean size comparisons between introgressed and native mussels were analysed using Ladysmith mussels only, since this site maintained a mussel population over the entire sampling period. Generally, the introgressed mussels remained significantly larger than the native mussels except in May, 1998. In January, 1999 a comparison could not be made as no native mussels were collected (Figure 1.5). In May, 1998, the mean size of the introgressed mussels was not significantly different from the native mussels ($p=0.102$). A decrease in mean size of the native mussels was also noted after July, 1998 (Figure 1.5). Large variation in the standard errors of the introgressed mussels was due to low sample numbers ($n_{4/98}$= 4 of 29, $n_{5/98}$= 3 of 43, $n_{6/98}$= 4 of 42, $n_{7/98}$= 7 of 48, $n_{8/98}$= 19 of 43, $n_{10/98}$= 22 of 39, $n_{1/99}$= 18 of 18, $n_{4/99}$= 11 of 21, $n_{5/99}$= 6 of 33).

Salinity and Temperature: Small Scale

Although no major differences in salinity values were noted, there were some differences in patterns of increases and decreases at each of the six sites (Figure 1.6). Many of the differences were noted at Ladysmith and at the outer Chemainus dock.

No major differences in temperature were noted over the sampling period (Figure 1.7). Gaps in the data set are due to the loss of temperature loggers during the sampling period. The Maple Bay data were approximated because the data logger that was initially lost at the beginning of the sampling period was found in the bay at the end of the sampling period; the depth of its recordings, however, was unknown.

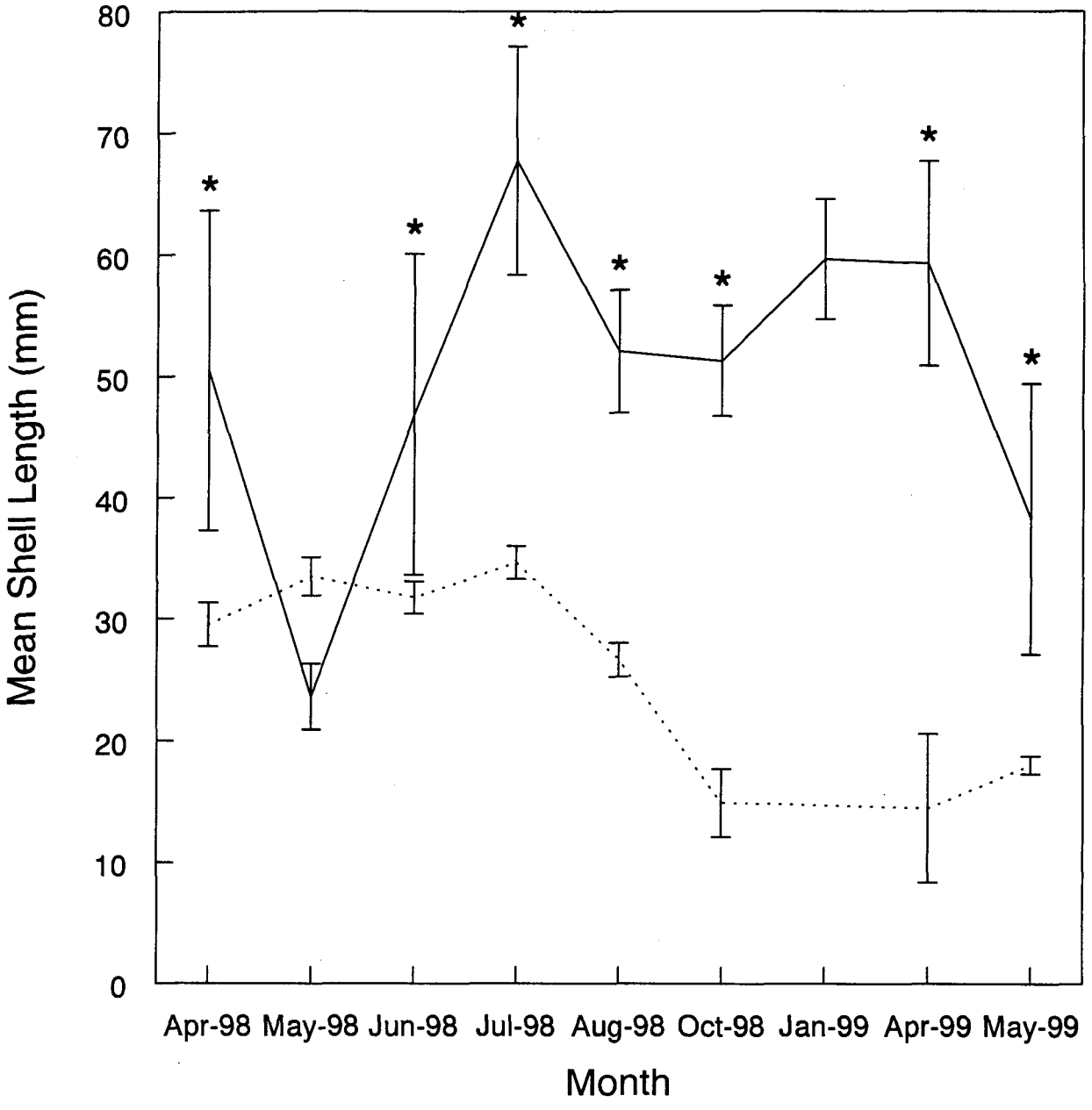


Figure 1.5. Temporal comparisons of mean shell length differences between introgressed (solid line) and native (dotted line) mussels from Ladysmith, B.C. (small scale sampling) between April, 1998 and May, 1999.

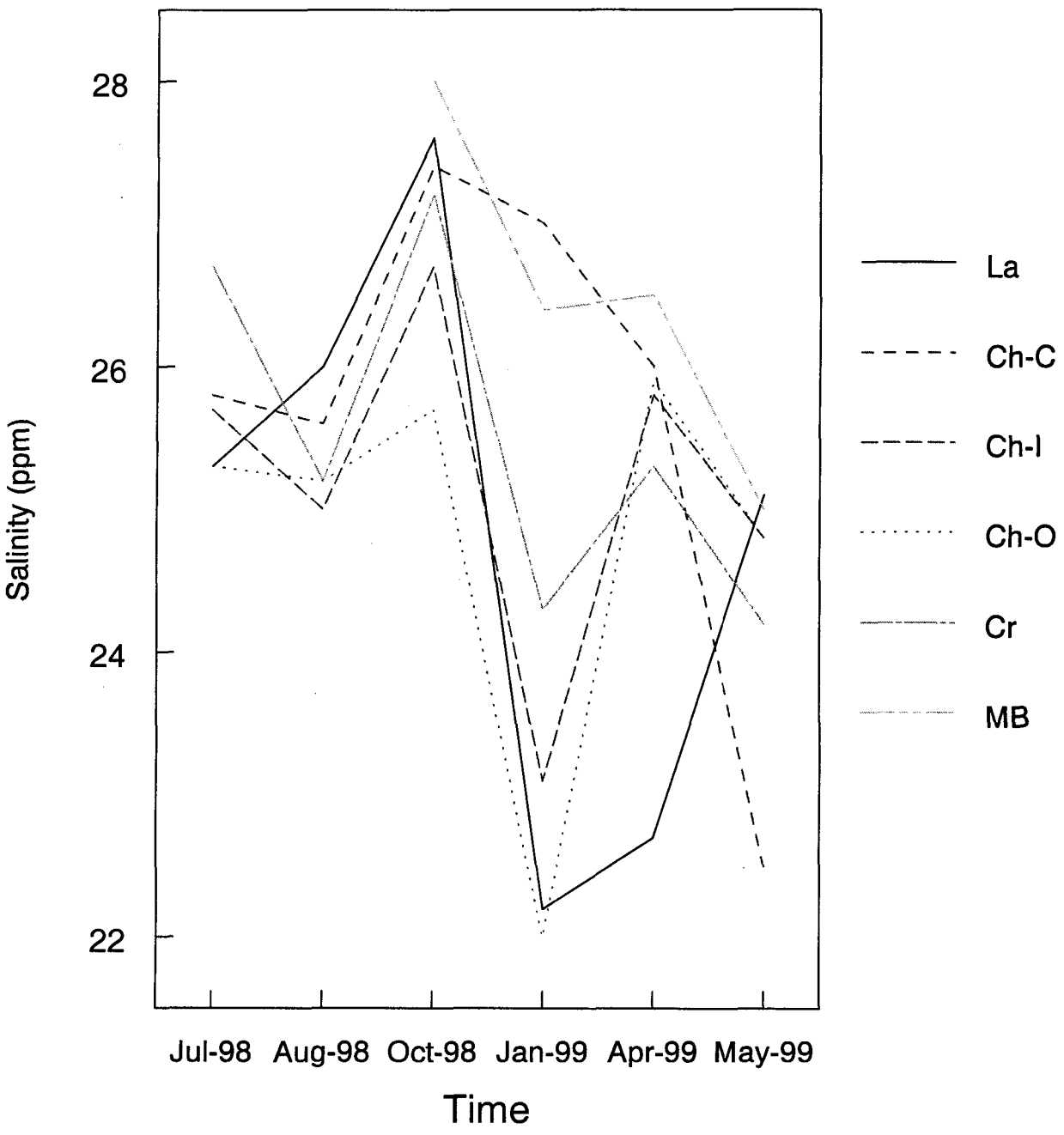


Figure 1.6. Temporal comparisons of salinity measurements taken from each of the six sites in the small scale sampling between July, 1998 and May, 1999. La – Ladysmith, Ch-C – Chemainus, commercial dock, Ch-I – Chemainus, inner dock, Ch-O – Chemainus, outer dock, Cr – Crofton, MB – Maple Bay.

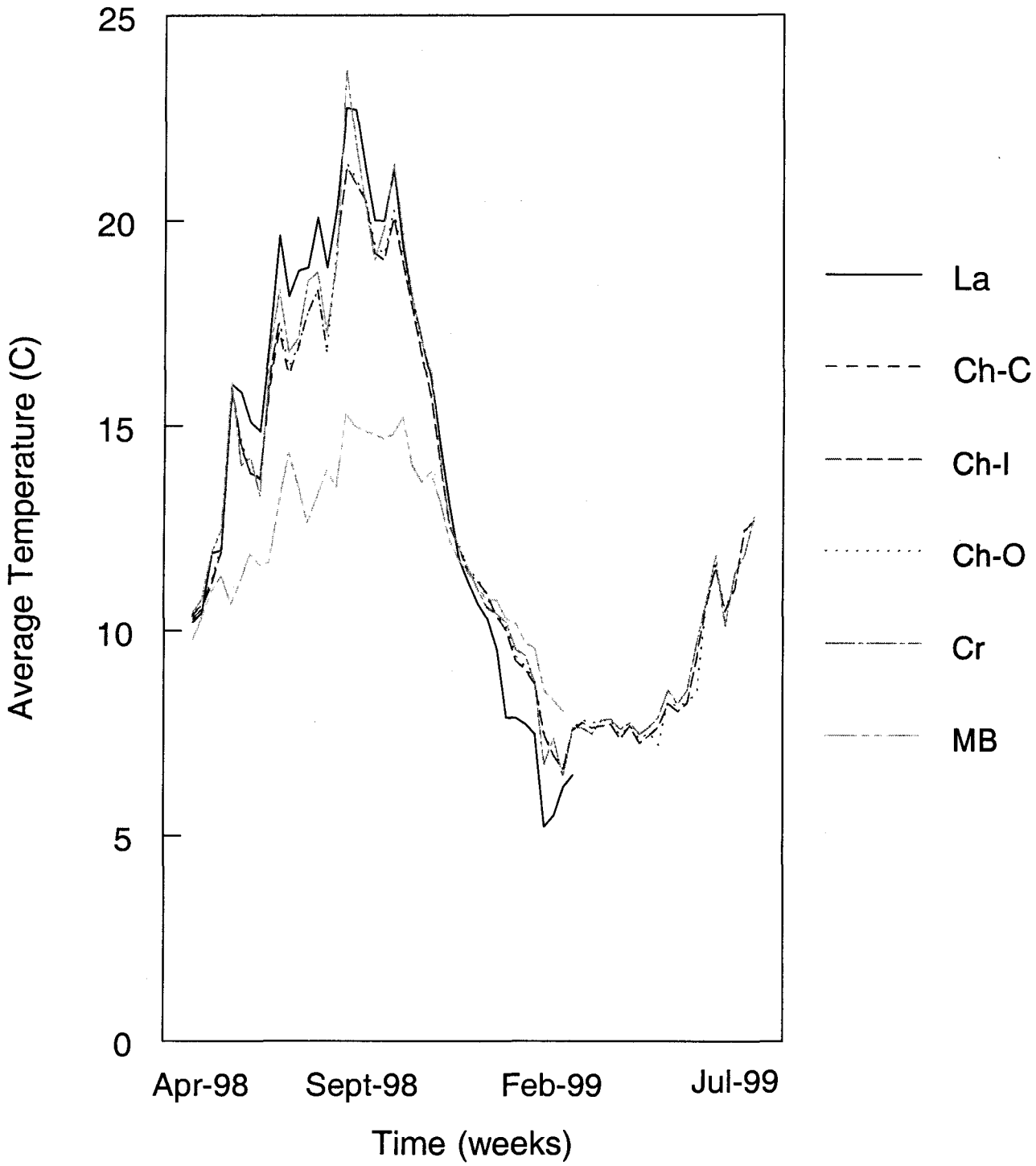


Figure 1.7. Comparison of average weekly water temperatures of each of the six small scale sampling sites over the course of the sampling period between April 3, 1998 and May 27, 1999. La – Ladysmith, Ch-C – Chemainus, commercial dock, Ch-I – Chemainus, inner dock, Ch-O – Chemainus, outer dock, Cr – Crofton, MB – Maple Bay.

1.5. Discussion

Temporal, spatial and genetic stability

The results of this study at both large and small scales indicate that *Mytilus edulis* and *M. galloprovincialis* (designated the "alien" mussels) have been introduced into British Columbia waters, have persisted, and are hybridizing with the native species, *M. trossulus*. The introduction of the alien mussels into Georgia Strait has been speculated to be the result of discharged ballast water (Heath et al 1995; Springer and Heath 2001) and/or immigration from mussel farms in Puget Sound, Washington. Additionally, recent importation of *M. edulis* and *M. galloprovincialis* stock for aquaculture purposes (pers. comm. D. Kieser, Fish Biologist, Fisheries and Oceans Canada) may represent an alternative introduction vector. Regardless of the vector of introduction, the alien mussels in Georgia Strait have potentially created a new hybrid zone on the east coast of Vancouver Island. It is apparent from this study, however, that this new hybrid zone is temporally, spatially and genetically unstable.

The temporal instability is most dramatically indicated by the crash of the introgressed population in 1996, most notably in Chemainus, followed by a gradual increase in numbers to 1998. Chemainus and Moses Point, however, were the only two sites that showed a significant change in introgression levels over sequential time intervals. Whether these changes indicated an actual shift in the location of the hybrid zone from Chemainus to Moses Point, or possibly temporal changes such as an unrelated crash in Chemainus coupled with an introduction in Moses Point, cannot be determined with these data. The spatial instability is indicated by the changes in the distribution of the introgressed mussels, mainly from the central, east-coast of the island to the south end. Although all sites showed the presence of introgressed alleles at some point in the five year sampling period, the greatest concentration occurred among the sites located between French Creek and Moses Point. Despite small changes, it is apparent that over the five

years of this study, there were shifts in the position of the hybrid zone in Georgia Strait. The genetic instability of the Georgia Strait hybrid zone was demonstrated by the variation in divergence from HWE among populations, and across time. The sites that were found to be significantly out of HWE were those along the central to south coast of the island. In all cases, the departure from HWE was due to significantly fewer hybrids than were expected.

Interestingly, considerable variation in introgression levels was observed between even the geographically adjacent sites, indicating a lack of uniformity at even the finest spatial scale employed for the annual sampling. The small-scale data clearly indicated that there is a highly patchy distribution of introgressed alleles on the east coast of Vancouver Island. The patchy distribution was noted not only on a 10-20 km scale, but even within a single bay (< 100 m). Generally such genetic differentiation has been speculated to be the result of environmental differentiation (such as temperature, salinity and wave exposure) and local adaptation by species; a "mosaic" type of hybrid zone. Microgeographic levels of genetic structuring have been previously shown in various marine invertebrates including prawns (Berglund and Lagercrantz 1983), barnacles (Bertness and Gaines 1993), corals (Hellberg 1996), copepods (Burton 1986) and mussels (McGrorty and Goss-Custard 1993; Sarver and Foltz 1993; Viard et al 1994; Bates and Innes 1995; Suchanek et al 1997; Pedersen et al 2000). In these cases, genetic structure among populations has been found along coastal areas as well as within individual bays. The results of the small-scale study clearly indicate that these mussels do not have a panmictic population structure. It is apparent at this scale that these mussels demonstrate genetic change over even very small spatial and temporal scales. The theory that, as broadcast fertilizers, mussel populations would not show any genetic structuring over small distances was not supported in Georgia Strait. Why is there genetic variation over such a small scale, since presumably larvae would disperse over this short distance? It may be the result of selection against hybrid individuals, inconsistent introductions of alien individuals and irregular environmental

conditions to which introgressed mussels are not suited. Small but notable differences in salinity were found among the six sampling sites (even between the three sites within Chemainus). Very little differences in temperature were noted among the six sites suggesting that salinity differences may be part of the reason for the genetic structuring. Clearly, the accepted paradigm of panmictic population structure in high dispersal, marine invertebrate species does not hold for all marine invertebrates. The data from this study show conclusively that blue mussels do not follow the paradigm.

Theoretical considerations

Previous studies have classified some of the *Mytilus* hybrid zones as mosaic zones (Sarver and Foltz 1993, Viard et al 1994; Gardner 1994b; Penney and Hart 1999; Rawson et al 1999). Based on the results of this study, however, it appears that the situation in Georgia Strait does not conform to any one existing hybrid zone model. A better model would be a combination of the mosaic hybrid zone model with ongoing immigration. Such an "immigrant mosaic model" encompasses the introduction of alien mussels, possibly sporadically, and potential inconsistencies in reproductive success due to low hybrid fitness (Springer and Heath 2001). The mosaic nature of the Georgia Strait hybrid zone would also be expected to reflect environmental differences among sites and over time. For example, the small-scale study indicated small, but notable differences in salinity among the six sites over time. As well, other data (Chapter 2) show differences in water temperature between Chemainus and YIAL (on Quadra Island). Both temperature and salinity differences can drive selective pressure in species and/or populations adapted to only a narrow range of environmental conditions (Brenko and Calabrese 1969; Hilbish and Koehn 1985). Thus, subtle environmental changes (as a result of global warming for example) could lead to a rapid change in the intertidal invertebrate species composition of Georgia Strait. For example, Nichols et al. (1990) monitored a species invasion by an

Asian clam (*Potamocorbula amurensis*) in San Francisco Bay. The invasion of this clam resulted from an increase in the salinity of the Bay (due to low river inflow and a previous flood that decimated the indigenous invertebrate community in the area) creating the appropriate conditions for colonization. The Asian clam was able to maintain its population size even after the salinity in the bay had returned to its lower levels, thereby permanently altering the benthic community at this site in San Francisco Bay (Nichols et al. 1990).

The immigration mosaic hypothesis would also account for the instability of the Georgia Strait hybrid zone. Sporadic introductions or immigration of alien mussels, which would only be successful given the right environmental conditions, may not provide a large enough population of alien mussels to reproduce successfully with native mussels. Thus, those introduced mussels capable of reproducing would likely fertilize native mussels and produce hybrids as opposed to maintaining a pure-type alien gene pool. Furthermore, the resulting hybrids that manage to reproduce would also likely mate with native mussels leading to backcross progeny. Differing spawning periods of the species, however, could act as a barrier to hybridization, as gametes from each species would not be in the water at the same time. This is not likely to be the case in Georgia Strait, however, as the majority of non-native mussels were either backcrosses or hybrids with few pure-types (either *M. galloprovincialis* or *M. edulis*) suggesting similar spawning times.

Given the potential temperature similarities between Georgia Strait and the east coast of Canada (and importation for aquaculture), *M. edulis* may prove more invasive in Georgia Strait and hybridize more readily with *M. trossulus*. *M. galloprovincialis*, however, was found at a higher frequency than *M. edulis* and more hybrids were of the *M. trossulus* – *M. galloprovincialis* type (data not shown). This suggests that despite *M. galloprovincialis* being generally found in warmer waters than *M. edulis* (Gosling 1992a) in Georgia Strait, *M. galloprovincialis* mussels are either able to migrate more readily into Georgia Strait, or are more likely to be in the ballast water of ships travelling into Georgia Strait. That is, the

introduction vector of *M. galloprovincialis* may be larger than *M. edulis*. An alternative hypothesis is that the *M. trossulus – galloprovincialis* hybrids have an increased fitness compared to *M. trossulus – edulis* hybrids. The *trossulus-galloprovincialis* hybrids could have a combination of *M. trossulus* alleles that are most suited to the temperature and salinity conditions of Georgia Strait, and the *M. galloprovincialis* alleles that provide strong byssal attachment to substrates (Willis and Skibinski 1992) which would be useful in the areas of fast moving water.

The immigration mosaic hypothesis leads to specific predictions for the size of the introgressed mussels relative to the native mussels. If the presence of alien mussels and hybrids were the result of intermittent introductions, the mean size of the introgressed population would increase over time. This is reflected in a cohort of introgressed mussels growing to larger sizes (possibly due to either faster growth or longer lifespan) with no new pure-type or introgressed juveniles being added to the population. This scenario requires that juveniles occur at a lower frequency due to the alien mussels being unable to successfully reproduce under the new environmental conditions. As a result, they would be unable to maintain a pure-type population or offer enough gametes for extensive fertilization with the native mussels for hybridization. Eventually, there would be a death of the introgressed cohort represented by a crash in numbers as well as in the mean size of introgressed mussels which would remain low until such time as another introduction occurred starting the cycle over again. This pattern appears to be present in my study. In 1994 and 1995 the introgressed mussels were significantly larger than the native mussels, however in 1996 there was no significant difference in size of the two classes which corresponded to the crash of the introgressed mussel population.

The departure from HWE in some samples, combined with extensive backcrossing is both interesting and confusing. Although a recent introduction of alien mussels would explain the departure from HWE as there would be pure-type, but too few hybrid

individuals, such a scenario would not be consistent with the numerous backcross and hybrid individuals in this study. Under the immigration mosaic hypothesis, though, unpredictable departures from HWE would be expected, as would the existence of hybrid and backcross individuals. Since the intensity of introduction would change over space and time, and the environment is known to be patchy and variable, a chaotic mosaic of HWE would result due to the combination of variable selective forces and introduction intensity. It may be that there is assortative mating occurring whereby the pure-types are preferentially mating with each other due to partial mating barriers, perhaps of a temporal nature. Furthermore, there may be strong genetic and environmental selection against the hybrids (Springer and Heath 2001), and, as a result, the sites within Georgia Strait show an inconsistent breakdown of HWE. Nevertheless, over time, it is possible that these sites will attain HWE, as those hybrids which do survive, will be likely to mate with *M. trossulus.* During successive generations, as the hybrids (backcrosses) continue to mate with pure native mussels, the genome will be progressively replaced with pure-type native alleles, resulting in weaker selection pressures after each generation. Over time, the selection pressure against introgressed mussels will decrease to the point where they can maintain HWE. Although the Wahlund effect may be contributing to the observed departures from HWE, it is unlikely to be a major factor here due to the broadcast spawning methods of the mussel species as well as the high dispersal capabilities of the pelagic mussel larvae. The Wahlund effect is a decrease in heterozygosity resulting from the mixing of individuals from genetically different groups (Johnson and Black 1984). Therefore, it is unlikely that sufficient population substructure exists within the sampling sites to allow a Wahlund effect as a possible explanation for the departures from HWE. The small-scale genetic analysis, however, indicates that considerable population substructure over even a very small spatial scale, thus a significant Wahlund effect, is a possibility that cannot be totally excluded.

Ecological Implications

It does not appear that the introgression of alien alleles is spreading substantially beyond the pre-crash levels in 1994 / 1995 except for the fluctuating levels of introgression at both the South Vancouver and West Vancouver sites of the mainland. This may indicate that the introgressed mussels are not a significant threat to the native mussels and ecosystems at this time. The presence of backcrossed mussels and the larger mean size of the introgressed mussels compared to the native mussels, however, could represent a future threat to the genetic make-up and ecological niche of the native mussels, *M. trossulus*. If, in the future, there was a significant change in environmental conditions within Georgia Strait that favoured the alien mussels, they could then maintain and expand a population. An increasing number of backcrossed individuals will mean that the alien alleles are introgressing further into the native population thereby disrupting the native gene pool. An increase in, and the maintenance of the average size of the introgressed individuals could result in a physical displacement of the native population whereby the *M. trossulus* mussels would either be preferentially selected by predators, due to their smaller size (Paine 1976; Bustnes 1998; Hamilton et al. 1999), or may be crowded out as juveniles (Wootton 1993).

Despite the moderately low level of introgression and the small number of hybrid individuals within Georgia Strait to date, there is still the potential for serious consequences of continued introduction of alien mussels. A study of the *M. trossulus – M. galloprovincialis* hybrid zone along the California-Oregon coast suggests that the lack of introgression between the two species may be the result of a limited contact history (Rawson et al 1999). Similarly, there may not have been sufficient time for the alien alleles to introgress substantially into the native mussel population in Georgia Strait. Unpredictable environmental and population dynamic changes, though, may, in the future, change the balance in favour of a successful invasion. In Georgia Strait, populations of introgressed mussels have been changing over time and space. This led to the conclusion that alien

mussels are either being introduced irregularly into Georgia Strait, are not always introduced under suitable environmental conditions or their resultant hybrids demonstrate reduced fitness. Apparently, the *M. galloprovincialis* mussels from Washington, Oregon and California do not appear to be extensively migrating up the Pacific Coast (Hilbish 1999). As well, Springer and Heath (2001) determined that the hybrids in Georgia Strait demonstrate reduced fitness. Thus, it appears that alien mussels are not able to reach or maintain stable population size in Georgia Strait, nor do sufficient numbers of hybrid mussels appear to be able to survive in the area to maintain a stable hybrid zone. As a result, the null hypotheses for this study were rejected. It is apparent that the hybrid zone in Georgia Strait is not stable and there is the existence of microgeographic genetic structuring in the mussel populations. This unstable hybrid zone does not fit into any one of the pre-determined hybrid zone types and it instead appears to be a mosaic type of hybrid zone combined with migration, termed an immigration mosaic zone in this study. As a result of this instability, the relative ecological risk of on-going introductions in Georgia Strait may not be significant until such time as there is a change in environmental conditions that is conducive to both alien and hybrid populations.

Chapter 2: Survival and growth of local and transplanted mussels (*Mytilus* spp.) in Georgia Strait: prospects for mussel aquaculture.

2.1. Abstract:

Economic pressures and consumer demand for fish and shellfish have led to a worldwide increase in aquatic species introductions for aquaculture purposes. Currently, on the west coast of British Columbia, the shellfish industry is expanding to include the culture of mussels. Production problems with the native species, *Mytilus trossulus*, however, have resulted in the consideration of importing non-native species (*M. edulis* and/or *M. galloprovincialis*) for culture. One potential drawback to the importation of species for aquaculture is local adaptation, or the adaptation of populations to local environments as the result of selection pressures. This study examined the potential of using native blue mussels (*M. trossulus*) for aquaculture in Georgia Strait, B.C. as well as the local adaptation of *M. trossulus* mussels on the east coast of Vancouver Island, B.C. Mussels were collected from two sites (Chemainus, B.C. and Quadra Island, B.C. at Yellow Island Aquaculture Ltd.) and reared in cages in a common environment which allowed for monitoring of individual survival and growth. No significant differences were found in growth rate and survival between native and introgressed blue mussels; however, interpretations of these data need to be made with caution due to the small sample size of introgressed mussels (n=5). Transplanted mussels were found to have a significantly higher mortality than local mussels suggesting the presence of local adaptation in populations of *M. trossulus*. There were no significant differences in shell length or relative growth rate between transplanted and local mussels. The results of this study demonstrate the existence of local adaptation in B.C. mussels and the potential importance that it may have for shellfish aquaculture.

2.2. Introduction:

A worldwide increase in species introductions for economic gain has resulted from the increasing demand on, and the resultant decline of, wild marine fish and shellfish stocks (Mann 1979; Chew 1990; Lipton et al. 1992; Robinson 1999). A non-indigenous species is generally chosen for introduction when it has desired characteristics for commercial production (e.g., faster growth rate, better survival) than a local species. Species such as the American and Pacific oysters (*Crassostrea virginica* and *C. gigas*) and various salmon species have a long history of being introduced into various parts of the world in attempts to establish new breeding stocks, and hence fisheries (Carlton 1992; Chew 1990; Ruiz et al. 1997; Elton 2000).

Although many species introductions may be of economic benefit, the introduction of a non-native species for commercial purposes has often been ecologically problematic. Generally, little thought is put into the potential impacts of these introductions, which could range from no noticeable impact, to the extinction of native and endemic species (Chew 1990; Carlton 1992). Potential impacts associated with such introductions include the inadvertent importation of non-target organisms (i.e., pathogens, predators and competitors) along with the organism of interest (Chew 1990; Carriker 1992; Lipton et al. 1992). There is also the risk of genetic impacts through hybridization with native species (Gaffney and Allen 1992). When aquaculturists are considering the importation of a non-native species, a balance between economic need and environmental priorities is a necessity.

Currently, on the Pacific coast of North America, the shellfish aquaculture industry is expanding into the culture of mussels. In the past, the blue mussel (*M. edulis*) industry of the east coast of Canada has generally dominated this market. This species has been considered the most marketable choice in North America, due to its ability to reach commercial size quickly and it's high survival to harvest size (Scarratt 1993). The economic

value of *M. edulis* as an aquaculture species on the west coast, would depend on various factors such as whether the species would display similar growth rates under different environmental conditions, and whether hybridization with local species and the resulting genetic introgression would lead to changes in growth or mortality rates. These are some of the issues that must be considered by west coast mussel aquaculturists.

An alternative to importing east coast mussels for B.C. mussel aquaculture would be to culture the local species (*M. trossulus*). This mussel is common intertidally from Oregon to Alaska (McDonald and Koehn 1988) and is morphologically similar to *M. edulis*. *M. trossulus* has a growth rate that would provide a commercially marketable product (Heritage 1983; Mallet and Carver 1995). Studies on the feasibility of mussel aquaculture in B.C. with *M. trossulus* (referred to as *M. edulis*), however, have encountered difficulties as a result of a dramatic increase in mortality of these mussels in the late summer-early fall of their second year when they are reaching marketable size (Heritage 1983, Bower 1989; Taylor et al. 1992). Explanations for this dramatic increase in mortality have included energy depletion (Emmet et al. 1987; Tremblay et al. 1998a) and elevated lysosomal activity following spawning (Tremblay et al. 1998b) resulting in metabolic stress; however, these explanations may pertain more to larger mussels that expend a larger portion of their energy resources on reproduction (Worrall and Widdows 1984). Increased mortality has also been linked with high temperatures (>20°C), although mainly during times of low food supply (Incze et al. 1980). Temperature is more critical for intertidal mussels because they are regularly exposed to air during tidal changes and thus are susceptible to desiccation (Tsuchiya 1983). Another reported cause of the increased mortality is haemocytic neoplasia, which is characterized by rapid growth of abnormal haemocytes that fill vascular spaces; the cause of this disease has yet to be reliably identified (Moore et al. 1991; Bower 1992). Incidence of this disease in mussels has been found to be much higher on the west coast of North America than on the east coast or in Europe (Cosson-Mannevy et al. 1984;

Rasmussen 1986; Elston et al. 1988; Bower 1989). Further study into the cause(s), and possible prevention, of this heightened mortality are required if B.C. mussels are to be considered a commercially viable option for growers.

An additional potential barrier to successful importation or transfer of species for aquacultural purposes is local adaptation of the target species to their native habitat. Thus, if the target species is locally adapted to its native habitat, individuals presumably won't be adapted to their new habitat unless it is similar. Local adaptation results from environmental pressures that drive selection of individuals exhibiting traits that enhance survival or reproductive success (Rawson and Hilbish 1991; Taylor 1991; Boersma et al. 1999). These selection pressures, resulting from differing environmental conditions, can also lead to genetic divergence of populations (Hilbish and Koehn 1985). Local adaptation has been reported in a range of organisms and systems, including plants (Kudoh and Whigham 1997), host-parasite systems (Gandon et al. 1996; Lively and Jokela 1996), phytophagous insects (Horton et al. 1991), *Daphnia* (Boersma et al. 1999), salamanders (Storfer et al. 1999), marine fish (Taylor 1991; Adkison 1995; Conover 1998) and various marine invertebrates such as prawns (Berglund and Lagercrantz 1983), mussels (Koehn et al. 1976; McGrorty and Goss-Custard 1993), barnacles (Bertness and Gaines 1993) and corals (Hellberg 1996). Local adaptation in marine invertebrates is particularly interesting because of their high larval dispersal capabilities. Such dispersal creates the potential for high levels of gene flow, thereby limiting opportunities for genetic differentiation, and hence local adaptation, in marine invertebrate populations (Burton 1983; Hedgecock 1986; Palumbi 1994). Genetic differentiation, however, can result when populations of marine invertebrates become adapted to their local environments through larval and/or adult selection based on environmental conditions such as temperature, salinity and physical structure and disturbances. The initial settlement of larvae in any one area may be heterogeneous, if individuals originate from a variety of areas. If specific individuals are

genetically able to survive local environmental conditions ("locally adapted"), however, they will thereby drive the genetic differentiation of the population (Koehn et al. 1976; Gartner-Kepkay and Zouros 1983; Heath et al. 1995; Hilbish 1985; Hilbish and Koehn 1985; Bertness and Gaines 1993; David et al. 1997; Schmidt and Rand 1999; Lewis et al. 2000). Thus local adaptations are an important factor to consider when aquaculturists attempt to transfer individuals among sites that differ environmentally.

Direct tests for local adaptation can be performed using reciprocal transplantation experiments. Individuals from two or more populations are collected and grown at each of the sites and monitored for growth, survival, physiological and reproductive differences. For example, Bertness and Gaines (1993) used transplantation studies to link thermal stress and flushing rates with local adaptation of acorn barnacle (*Semibalanus balanoides*) populations. Koehn et al. (1976) examined variation at the *lap* locus (which is involved in the regulation of internal salinity concentrations of mussels) among populations of *M. edulis* and found that one allele (lap^{94}) was selected against in the more estuarine environments (lower salinity) and selected for in the more oceanic sites (higher salinity). Studies done on *M. edulis* in various parts of the world also found that local adaptations such as site-environmental interactions were a large determinant in growth (Dickie et al. 1984; Mallet and Carver 1989; Johannesson et al. 1990; Kautsky et al. 1990; Stirling and Okumus 1994) and mortality differences (Mallet et al. 1987; Stirling and Okumus 1994). Kautsky et al. (1990) found that when mussels from Alaska were transplanted to the North Sea, they were preferentially predated by sea stars. They proposed that with few predators in Alaska, the mussels had evolved to have high fecundity but thinner shells and smaller abductor muscles. Thus, when transplanted to the North Sea, they were more easily predated than the local mussels, resulting in preferential predation (Kautsky et al. 1990). Local adaptation clearly occurs in marine invertebrates, despite high potential larval dispersal, and this must be taken into account when choosing stock for commercial culture.

The purpose of this study was to examine the commercial potential of using native blue mussels (*M. trossulus*) for aquaculture in Georgia Strait, B.C. The growth rates and survivorship of *M. trossulus* were compared to those of non-native/ introgressed mussels (*M. edulis* and *M. galloprovincialis*, hybrids and backcrosses); with the hypothesis that pure *M. trossulus* would be inferior overall to introgressed blue mussels based on prior performance studies of *M. edulis and M. galloprovincialis*. Second, a comparison of growth and survivorship between local *M. trossulus* mussels from Quadra Island (Yellow Island Aquaculture Ltd. (YIAL)) and *M. trossulus* mussels transplanted from Chemainus (CH), B.C. was made at YIAL. It was hypothesized that the local (YIAL) mussels would perform better than the transplanted (CH) mussels, under the expectation of local adaptation.

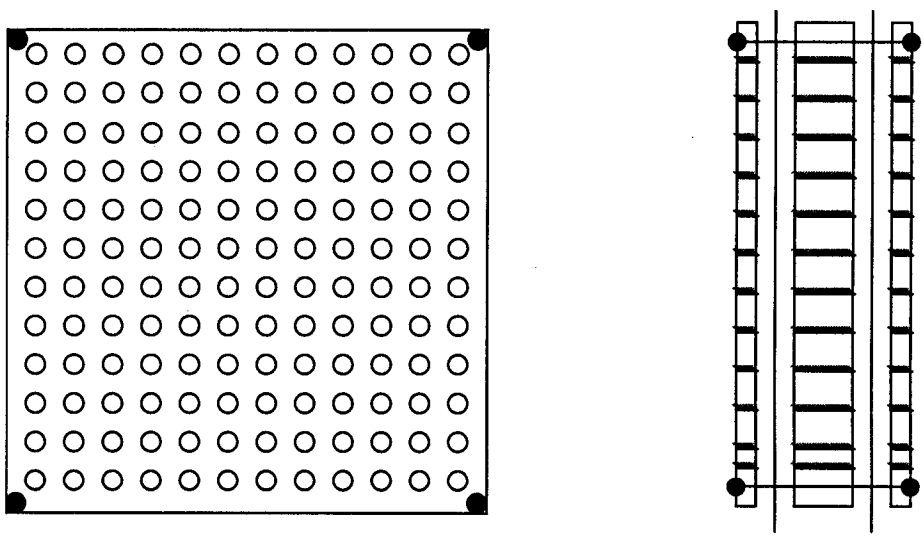
2.3. Materials and Methods:

2.3.1. Rearing

As juvenile mussels were selected for the experiment, a method of marking or following the individual mussels was required. Such a technique would enable the comparison of differences between native mussels (*M. trossulus*) and introgressed mussels (*M. edulis, M. galloprovincialis* and hybrids), which would be genotyped at the end of the experiment. Many shellfish growth experiments use stressful marking procedures such as engraving (Dolmer 1998), filing (Dehnel 1956) or scouring (Harger 1970b) numbers into the shell, in order to follow individuals, although these methods are not suitable for small mussels. Cutting numbers into shells was also found to correspond to increased infestation of the mud-worm, *Polydora websteri*, in mussels (Freeman and Dickie 1979). Growth experiments may also require the breakage of byssal threads in order to measure individuals (Freeman and Dickie 1979). Although Freeman and Dickie (1979) found no effect of byssal thread breakage on growth and survival of mussels, the study only considered larger (25 – 31 mm) mussels. As a result, an alternative method was devised

for this experiment to avoid such physiological stresses. A mussel cage was designed to keep individuals physically separate, and thus allowed the monitoring of individuals without having to physically mark them.

The cages were layered units consisting of a 60x60x5 cm center polyethylene square, 0.5 mm mesh screen on either side of the center square and 60x60x1 cm polyethylene squares on each outer side. One hundred and forty-four 5.0 cm holes (in a 12 X 12 grid) were drilled through the polyethylene layers to house individual mussels. The screen was used to keep the mussels in their individual holes for identification purposes while allowing free water flow to provide oxygen and food for the mussels. The layers were held together with stainless steel bolts and a weight was attached to the bottom of each cage, and ropes and clips to the top for suspension (Figure 2.1). The cages were suspended 1 m below the surface from a log anchored in 10 m of water approximately 500m outside of the YIAL lease.



Front View Side View

Figure 2.1. Schematic diagram of the mussel cage. Front view shows the location of the 144 mussel holes, side view shows the layering of the polyethylene and mesh screen. The solid black dots represent the bolts that held the cages together. The shadowed lines represent the holes going through the polyethylene layers.

2.3.2. Sample Collection and Transport

From March 20 to April 4, 1998, small mussels (less than 10 mm and thus settled in spring 1998) were collected from two sites for use in these experiments. On the first collection date (March 20, 1998), mussels were taken from Yellow Island Aquaculture Ltd. (YIAL) and Chemainus (CH) and used to fill the YIAL and CH1 cages (Appendix III). The CH2 cage was filled on March 28, 1998 and the CH3 cage was filled on April 4, 1998. The Chemainus samples were collected over the course of three weeks to maximize the likelihood of collecting introgressed (*M. edulis*, *M. galloprovincialis*, hybrids and backcrosses) mussels. Each cage was initially stocked with 144 mussels (1 per hole). The YIAL collection sites were subtidal anchor ropes in the waters surrounding a fish farm on Quadra Island near Campbell River, B.C. (Figure 2.2). The YIAL mussels were gently pulled off the anchor ropes at YIAL, placed in a container of seawater, taken to shore, measured and placed in individual compartments in the YIAL cage (approximate transport and handling time: 30 minutes). The CH collection site was on the outer edge, subtidal surface of the government wharf in Chemainus, B.C. (Figure 2.2). The Chemainus site was chosen because previous work had shown that Chemainus had the greatest abundance of introgressed blue mussels (*Mytilus* spp.) on the east coast of Vancouver Island (Heath et al. 1995). The CH collected mussels were gently pulled off the subtidal portion of the dock, placed in a container of seawater on ice, taken to YIAL, measured and placed in individual compartments in the cages (approximate transport and handling time: 4 hours). The cages were suspended from a chain on a log anchored in 10 m of water approximately 500 m outside the YIAL lease and hung at a depth of 1m below the surface.

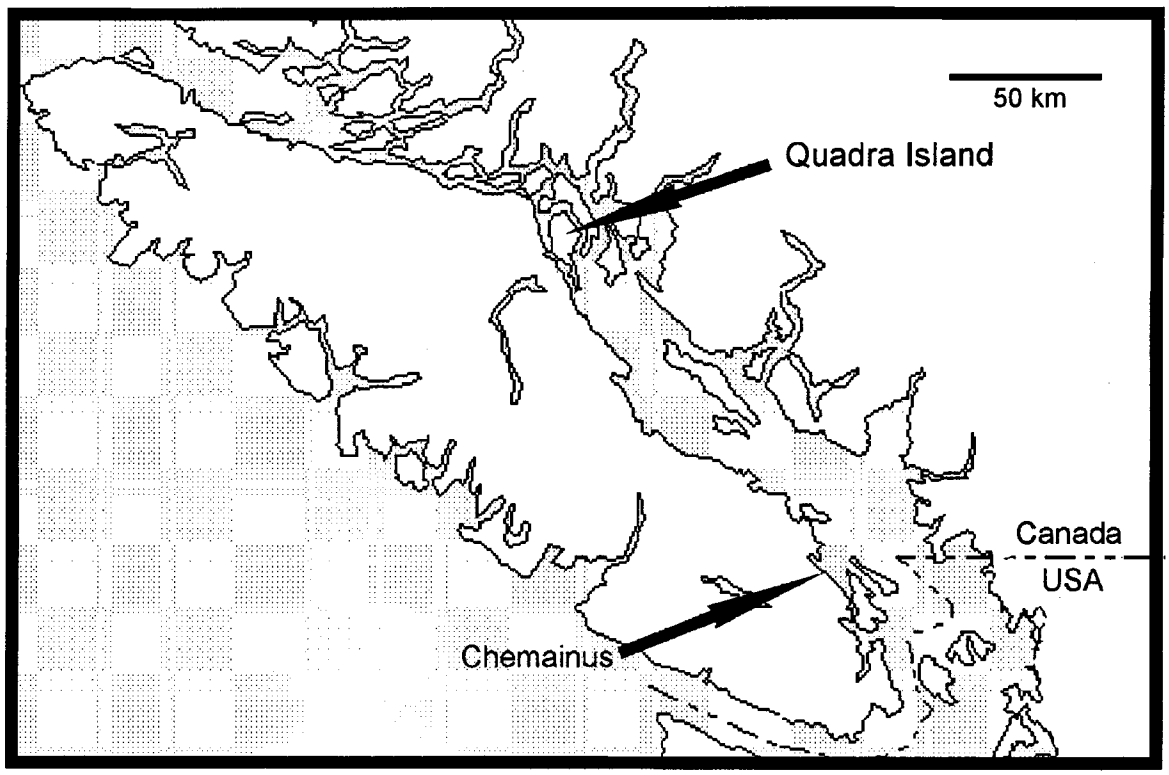


Figure 2.2. Map of Vancouver Island, British Columbia. Yellow Island Aquaculture Ltd. (local collection) is located on the west central coast of Quadra Island. Chemainus is the collection site for the transplanted mussels.

2.3.3. Survival and Growth Monitoring:

Mussel survival and growth was monitored throughout the experiment (March 20, 1998 – July 15, 1999); however, more frequent monitoring occurred during the summer months (Appendix I). When cages were retrieved, one cage was opened at a time and the others were held in saltwater to minimize desiccation. Sampling involved recording the position of all mussels, whether they were alive or dead (mortality check), and removing each individual from its individual compartment and measuring total shell length (from the tip of the beak to the posterior edge) with digital calipers (± 0.1 mm). Live animals were identified by their response to being removed from the water, or by gently probing them. If the mussel was alive, it was returned to its compartment. If the mussel was dead, it was measured and preserved in 95% ethanol. Shell length was not measured on all dates to reduce disturbance to the mussels. Before being returned to the water, all cages were cleaned by removing fouling organisms.

Initially, the mussels were checked every week for survivorship and every three weeks for total length (Appendix III). Unfortunately, from all cages, there was approximately 10% sampling mortality as a result of mussels that were damaged when the mussel cages were opened. These sampling mortalities were removed from all subsequent analyses. Weekly mortality checks were minimized to limit the number of times the cages were opened (and hence the sampling mortality). During the summer dates, mortalities were only removed when the mussels were being measured. During the fall and winter, the mussels were measured and checked approximately every 6 to 8 weeks. Temperature was monitored throughout the growth period at YIAL and Chemainus with a temperature data logger (Onset Computer Corp., Pocasset, MA).

2.3.4. Specimen Identification:

The genetic analysis protocol described in Chapter 1 was used in this study to identify each mussel to species at two marker loci. Briefly, mantle tissue from each mussel was digested overnight in proteinase K, and the DNA was extracted using a phenol extraction protocol for polymerase chain reaction (PCR) (Heath et al. 1995). DNA fragments were PCR amplified using the ITS protocol described in Heath et al. (1995) and the Glu-5' protocol described in Rawson et al. (1996) (see Chapter 1). Individual mussels were scored for genotype, as described in Chapter 1, at each marker locus on the basis of diagnostic RFLPs (ITS; Heath et al., 1995) and using an automated DNA sequencer to determine PCR-FLVs (Glu-5'; Rawson et al., 1996). Mussels were identified as being native (*Mytilus trossulus*), or introgressed *(M. edulis* or *M. galloprovincialis*, hybrid or backcross).

2.3.5. Experiment 1: Native vs. Introgressed Mussels.

In this experiment, all collected mussels (YIAL, CH-1, CH-2, CH-3) were included to maximize the number of introgressed genotype mussels. Mussels in the three CH cages were collected over three successive weeks in an attempt to capture more introgressed mussels for comparison against native mussels.

A comparison could not be made between all the native and introgressed mussels because there was an insufficient number of introgressed mussels collected. Each individual mussel identified as introgressed (*Mytilus galloprovincialis* or *Mytilus edulis*, hybrid or backcross) was compared to two randomly chosen, neighboring native (*M. trossulus*) mussels. Introgressed mussels were compared to neighboring mussels in order to minimize potential cage effects. Relative growth rate (RGR) (%/day) was calculated as:

$$RGR = (SL_2 - SL_1)/(SL_1 \bullet t) \qquad \text{(Eqn. 2.1)}$$

where SL_1 is the shell length of an individual at time 1 and SL_2 is the shell length of the same individual at time 2 and t is the number of days between the two dates. Comparisons of the RGR and shell length (mm) of the introgressed mussels versus the native mussels were made from March 20, 1998 to July 31, 1998. Comparisons were not conducted after July 31, 1998, due to insufficient numbers of introgressed mussels, for meaningful statistical analysis because of the ongoing mortality of the mussels. One-way ANOVAs (Systat 7.0: Statistics 1997) were used to test for differences in shell length and relative growth rate between the two groups. Shell length was compared on each measurement date and relative growth was compared for each interval (from one measurement date to the next). Growth rates were arcsine square root transformed as suggested by Zar (1996) for ratio data.

2.3.6. Experiment 2: Local vs Transplanted Mussels.

Only mussels identified as native (*M. trossulus*) using molecular genetic markers were used to compare performance between local and transplanted mussels as a test of local adaptation (introgressed mussels were not included in the analysis). Only the YIAL (local) and CH1 (transplanted) cages were used in this analysis since they were collected and transported on the same date (March 20, 1998). Growth and survival were chosen as indicators of performance. For the comparison of shell length and growth rates, only those mussels that survived the entire duration of the experiment (March 20, 1998 to July 15, 1999) were used. Total shell length (mm) and RGR (Eqn. 2.1) of the transplanted mussels (CH1) were compared with that of the local mussels (YIAL). One-way ANOVAs were used to determine whether a significant difference ($p<0.05$) in shell length existed between the groups for each measurement date and in RGR for each interval. RGR was arcsine square root transformed as suggested by Zar (1996) for ratio data.

Percent mortality ([number of mussels dead for a given interval/ total number of mussels at beginning of interval] x 100) was determined for the local and transplanted groups for each interval. A 2 x 2 contingency table was used to test for differences in mortality on the interval showing the greatest mortality (July 31, 1998 to August 21, 1998) to determine if a significant difference between the two groups in the number of mortalities over this interval existed. Percent cumulative mortality (total number of mussels dead to a given date / number of mussels alive at the beginning) was calculated for the entire time period for each group. A 2 x 2 contingency table was used to determine if there was an overall difference in total mortality between the local and transplanted mussels over the course of the experiment.

2.4. Results:

2.4.1. Experiment: 1 Native vs. Introgressed Mussels.

A total of 313 mussels from the cages were successfully genotyped using PCR-FLVs and RFLPs. Of these, only 7 mussels were determined to be introgressed. Of these 7, only 5 were used in the growth and mortality analysis because one was from the YIAL cage and was not mixed with those from the CH cages, and the other was killed when the cage was opened and therefore had incomplete data. No significant differences (Table 2.1) in shell length or relative growth rate were found between the identified introgressed mussels and their neighboring native (*M. trossulus*) mussels (Figure 2.3). A repeated measures test that was used with the data, as a methods comparison, also indicated no significant differences in shell length or relative growth rate. Growth was only examined until July 31, 1998 as subsequently there were too few introgressed mussels for meaningful comparisons. Mortality could not be compared due to the low number of introgressed mussels.

Table 2.1. Summary of ANOVA results for native versus introgressed (*Mytilus*) growth experiments of shell length and relative growth rate (RGR). Where df – degrees of freedom, MSEf – mean square effect, MSE – mean square error, F – F-statistic, *p* – probability value.

Measurement	Date/Interval	df	MSEf.	MSE	F	*p*
Shell Length	April 18, 1998	1	2.754	3.887	0.709	0.415
	May 9, 1998	1	3.598	5.037	0.714	0.413
	May 30, 1998	1	2.977	5.373	0.554	0.470
	June 20, 1998	1	0.383	2.745	0.140	0.715
	July 11, 1998	1	0.208	1.674	0.124	0.730
	July 31, 1998	1	0.220	1.703	0.220	0.647
RGR	March 20 – April 18, 1998	1	0.000	0.003	0.027	0.873
	April 18 – May 9, 1998	1	0.000	0.001	0.594	0.455
	May 9 – 30, 1998	1	0.000	0.000	0.507	0.489
	May 30 – June 20, 1998	1	0.001	0.001	1.502	0.242
	June 20 – July 11, 1998	1	0.001	0.000	1.679	0.218
	July 11 – 31, 1998	1	0.000	0.001	0.021	0.886

2.4.2. Experiment 2: Local vs transplanted mussels.

Of the 142 *M. trossulus* in the YIAL cage (local) and 142 in the CH1 cage (transplanted), 35 and 47 mussels respectively were accidentally killed during the experiment and were removed from the analyses. In the YIAL and CH1 cages, 64 and 37 mussels respectively survived until the end of the experiment (July 15, 1999); these individuals were used in the growth/size analysis.

Although the transplanted mussels were slightly larger on each date, they were not significantly larger than the local mussels [after Bonferonni correction] (Figure 2.4b). Table 2.2 summarizes the ANOVA results for the RGR and size comparisons between the local and transplanted mussels and shows the dates on which the local mussels were marginally significantly larger before Bonferonni correction. Marginally significant results have been defined as results that are statistically significant before Bonferonni correction, but are

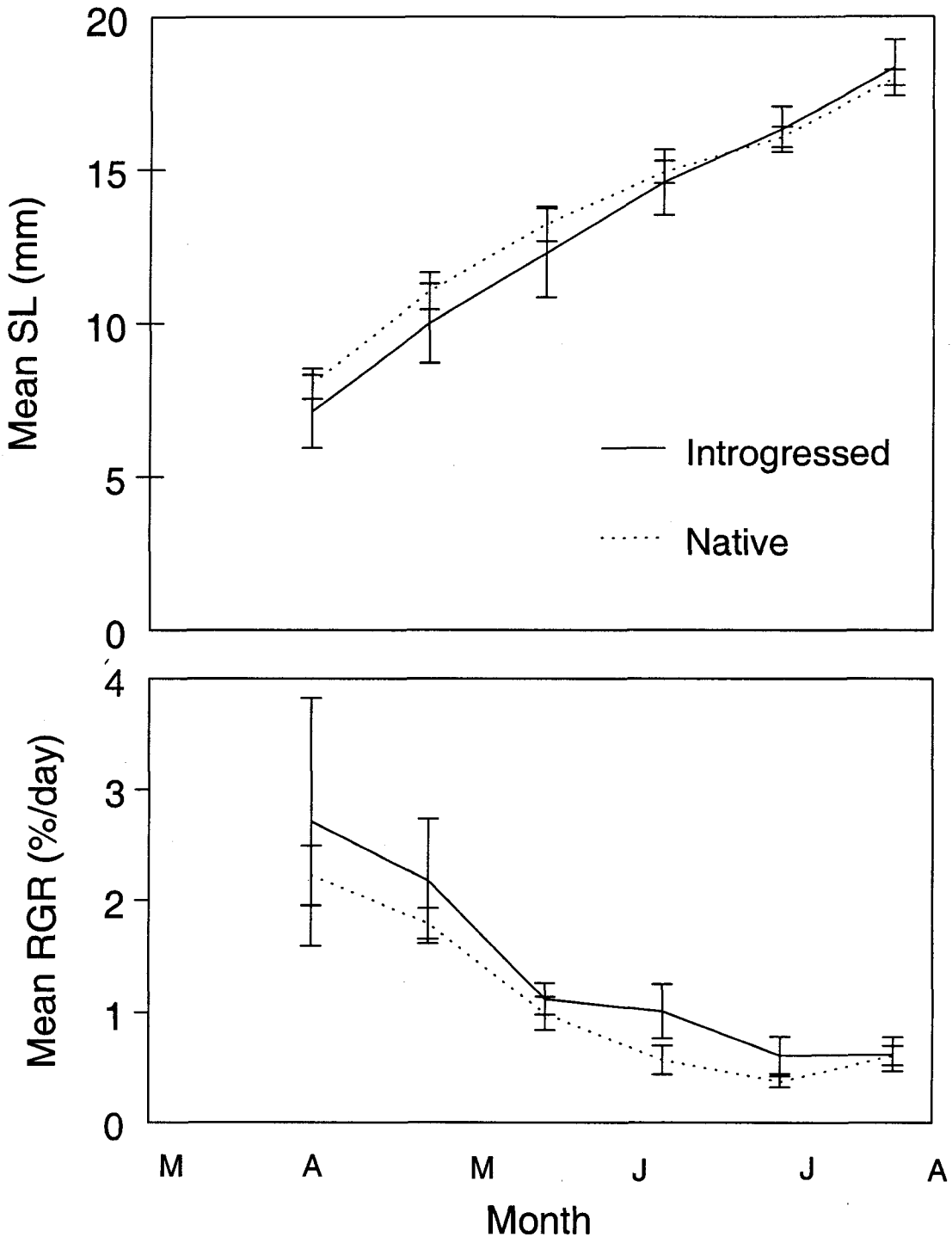


Figure 2.3. Mean shell length (SL) and relative growth rate (RGR) for introgressed (solid line) and native (dotted line) mussels from March 20, 1998 to July 31, 1998. The upper panel is the mean shell length of introgressed mussels (n = 5) compared to neighboring native (n = 10) mussels. Lower portion is the mean relative growth rates of introgressed mussels (n = 5) compared to neighboring native mussels (n = 10). There were no significant differences for either parameter (ANOVA, $\alpha \geq 0.05$) (Table 2.1).

rendered non-significant after Bonferonni correction (Ferguson 1996). The Bonferonni correction is a conservative test that decreases the chance of type I errors. Thus, those results that really are significant may be rendered insignificant by this correction (marginally significant result). Again, a repeated measures analysis was used on the data as a methods comparison. This analysis also indicated no significant difference in mean shell length between the two groups after Bonferonni correction. The greatest increase in mean shell length (Figure 2.4b) corresponded to higher water temperatures at YIAL during the summer months between June and August (Figure 2.4a).

Relative growth rate did not differ significantly (Figure 2.4c) between the local and transplanted mussels after Bonferonni correction (Table 2.2). The local mussels did have a marginally significantly higher growth rate than the transplanted mussels during two intervals: June 20 1998 to July 11 1998 and July 31 1998 to August 21 1998. Repeated measures analysis also gave a no significant result in RGR between the local and transplanted mussels. The decrease in RGR from the beginning of the experiment (March) until August coincided with the increases in water temperatures at YIAL (Figure 2.4a). The low relative growth rate during the fall and winter months matched lower water temperatures (Figure 2.4).

Of the 108 *M. trossulus* mussels in the local (YIAL) and 95 in the transplanted (CH1) cage at the beginning of the experiment (excluding those that were crushed), 41.7% and 61.1% respectively had died by the end of the experiment (July 15, 1999) (Figure 2.5b). The total number of mortalities at the end of the experiment (YIAL – 44, CH1 – 58) was significantly greater in the transplanted mussels than the local mussels ($\chi^2 = 7.935$; $p = 0.005$). The greatest mortality observed for a single interval occurred between July 31, 1998 and August 21, 1998 when 23% of the local mussels and 43% of the transplanted mussels died (Figure 2.5c). The "outbreak" mortality during this interval for the local and

transplanted mussels was found to be significantly greater in the transplanted mussels than in the local mussels ($\chi^2 = 5.852$; $p = 0.016$).

A comparison of water temperatures indicated that Chemainus had generally higher temperatures over the spring and summer (mid-April to the beginning of October) months than YIAL (Figure 2.4a, 2.5a). Water temperatures were similar at the two sites between October to April (Figure 2.4a, 2.5a).

Table 2.2. Summary of ANOVA results for *Mytilus trossulus* growth comparison between local and transplanted mussels. Where df – degrees of freedom, MSEf – mean square effect, MSE – mean square error, F – F-statistic, *p* – probability value.

Measurement	Date/Interval	df	MSEf	MSE	F	*p*
Shell Length	March 20, 98	1	3.98	5.26	0.76	0.39
	April 18, 1998	1	3.23	9.18	0.35	0.56
	May 9, 1998	1	23.40	9.68	2.42	0.12
	May 30, 1998	1	27.97	7.57	3.69	0.059
	June 20, 1998	1	41.59	6.62	6.29	0.014
	July 11, 1998	1	25.90	5.72	4.45	0.038
	July 31, 1998	1	33.5	5.73	5.85	0.018
	August 21, 1998	1	15.10	5.93	2.55	0.12
	October 30, 1998	1	21.88	6.58	3.33	0.072
	February 25, 1999	1	21.99	7.13	3.08	0.083
	April 22, 1999	1	21.07	7.00	3.01	0.087
	May 28, 1999	1	22.55	7.13	3.16	0.079
	July 15, 1999	1	17.18	7.09	2.42	0.12
Mean RGR	March 20 – April 18, 1998	1	0.003	0.001	2.15	0.15
	April 18 – May 9, 1998	1	0.001	0.001	2.08	0.15
	May 9 – 30, 1998	1	0.000	0.001	0.26	0.61
	May 30 – June 20, 1998	1	0.000	0.001	0.28	0.60
	June 20 – July 11, 1998	1	0.002	0.000	4.32	0.041
	July 11 – 31, 1998	1	0.000	0.000	0.26	0.61
	July 31 – August 21, 1998	1	0.002	0.000	4.98	0.029
	August 21 – October 30,	1	0.000	0.000	2.08	0.15
	October 30 – February 25,	1	0.000	0.000	0.056	0.81
	February 25 – April 22, 1999	1	0.000	0.000	0.005	0.95
	April 22 – May 28, 1999	1	0.000	0.000	0.13	0.72
	May 28 – July 15, 1999	1	0.000	0.000	1.50	0.23

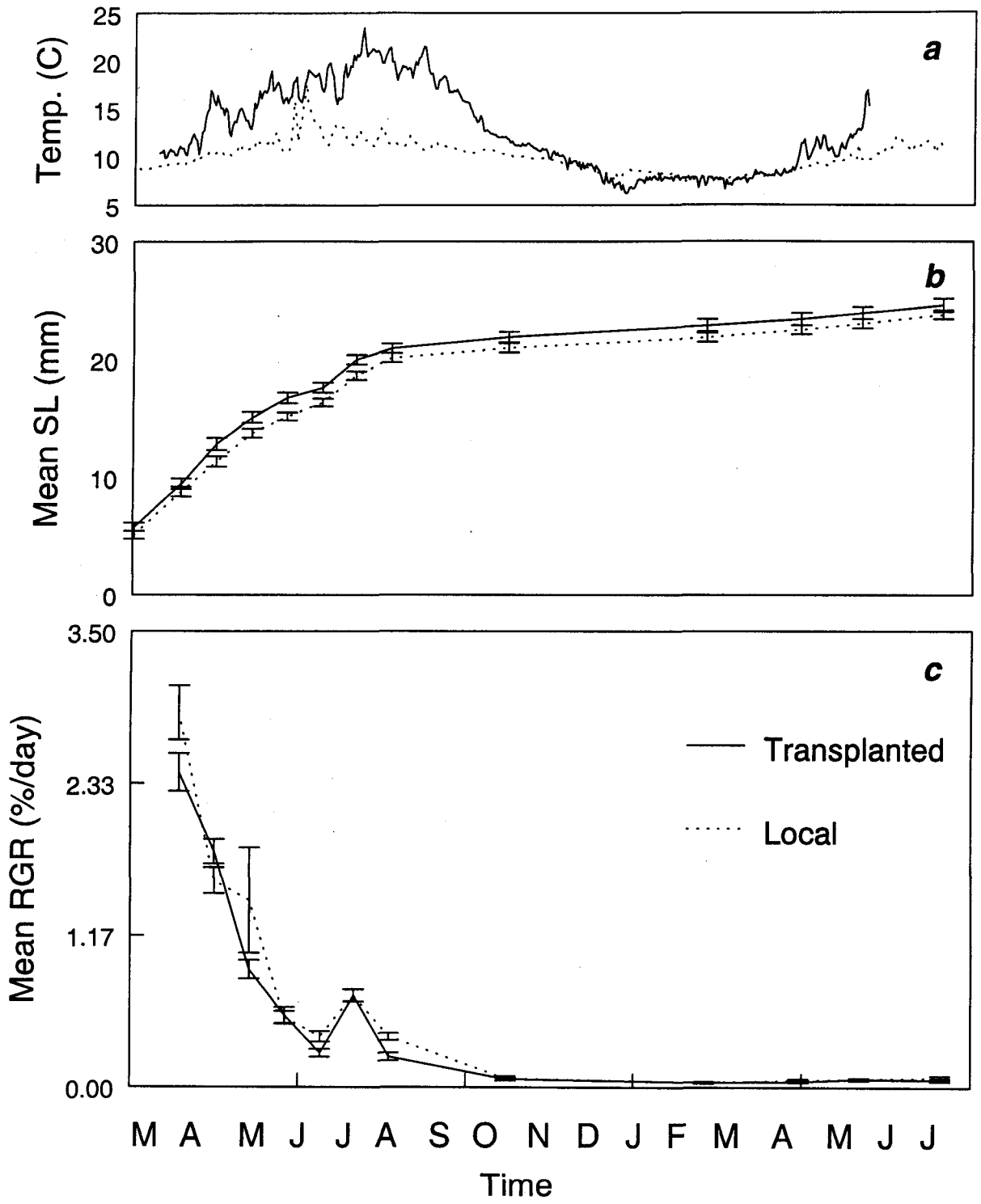


Figure 2.4. Mean daily temperatures (°C), shell length (SL) and relative growth rates (RGR) of the local (dotted line) and transplanted (solid line) mussels for March 20, 1998 to July 15, 1999. Panel *a* is the mean daily temperature profile for YIAL (dotted line) and Chemainus (solid line) at 1 m and 0.5 m depths respectively. Panel *b* displays the mean SL of all transplanted (CH1) and local (YIAL) *Mytilus trossulus* mussels. Panel *c* shows mean RGR of all transplanted (CH1) and local mussels (YIAL).

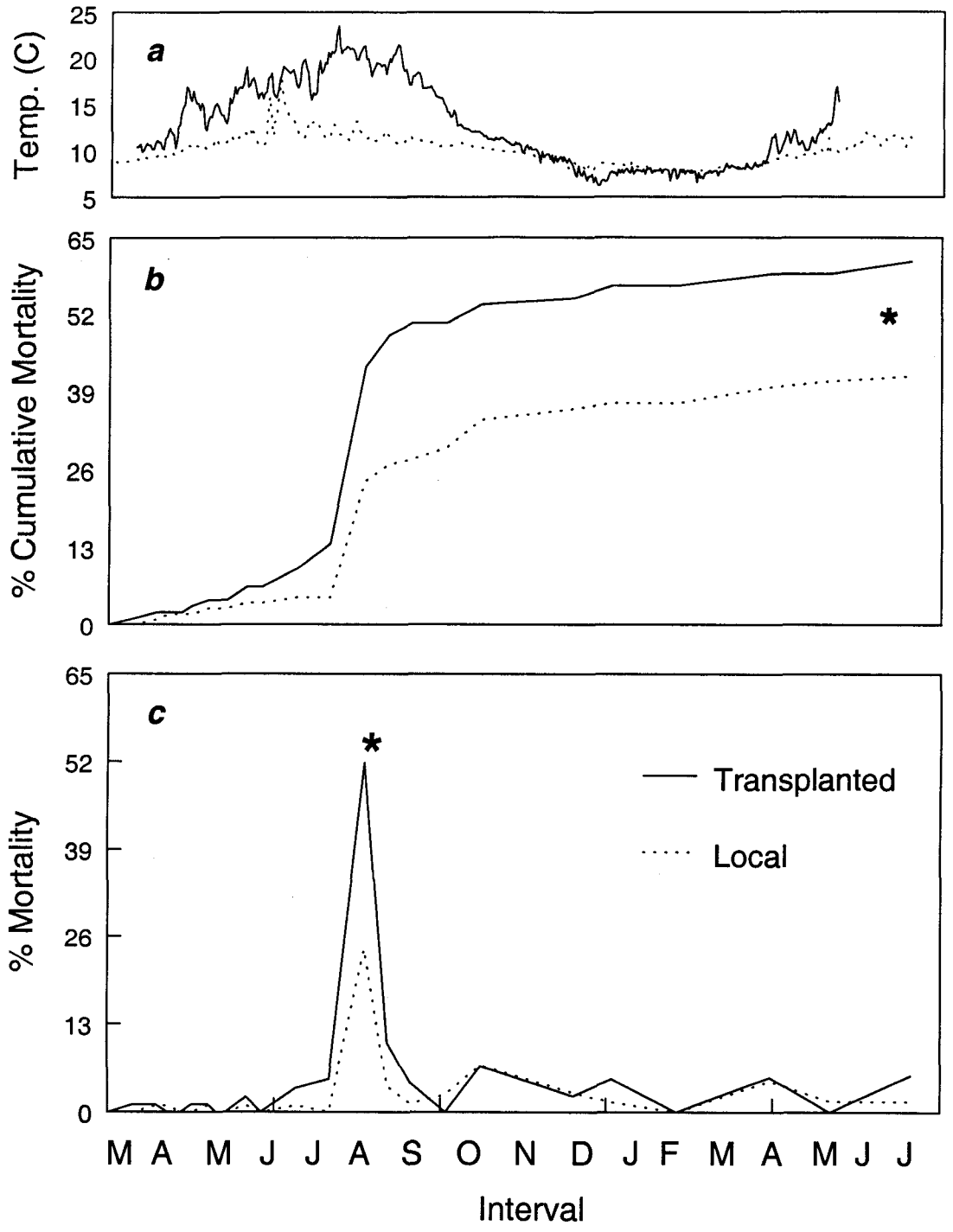


Figure 2.5. Mean daily temperature (°C), percent (%) cumulative mortality and percent (%) interval mortality of the local (dotted line) and transplanted (solid line) mussels for March 20, 1998 to July 15, 1999. Panel *a* is the water temperature profile for YIAL (dotted line) and Chemainus (solid line) at 1m and 0.5 m depths respectively. Panel *b* is the percent cumulative mortality of transplanted (CH1) and local (YIAL) *M. trossulus* mussels monitored. Panel *c* shows the percent mortality at each interval of transplanted (n_i = 95) and local (n_i = 108) *M. trossulus* mussels. (* denote significant difference after Bonferonni correction).

2.5. Discussion:

2.5.1. Experiment 1: Native vs Introgressed Mussels.

No significant difference in growth was found between the native and introgressed mussels (Figure 2.2); these results, however, should be interpreted with caution since they are based on only 5 introgressed mussels. Past studies have given conflicting results when comparing growth among the *Mytilus* sibling species. For example, *M. galloprovincialis* and the hybrids were found to grow significantly faster than *M. edulis* at the larval stage (Beaumont et al. 1993) and in intermediate ages (5-9 years) (Gardner et al. 1993). Introgressed mussels were also found to be larger on average than native mussels (*M. trossulus*) within Georgia Strait, B.C. (Chapter 1; Springer and Heath 2001). In a study by Skibinski and Roderick (1989), though, no significant difference in growth rate was found between *M. galloprovincialis* and *M. edulis.* A study comparing *M. trossulus* and *M. edulis* in Atlantic Canada found that *M. edulis* grew significantly faster than *M. trossulus* in a 45-55 mm size class, but no significant difference was found in a smaller size class (20-30 mm) (Mallet and Carver 1995). Mallet and Carver's (1995) study was one of the few studies that examined growth specifically in *M. trossulus.*

Differences in mortality between *M. trossulus* (native) and introgressed mussels could not be determined in this study due to insufficient numbers of introgressed individuals. Furthermore, predicting natural mortality differences is difficult due to the higher incidence of summer mortality in some mussel species or in some areas (Cosson-Mannevy et al. 1984; Elston et al. 1988; Bower 1989; Moore et al. 1991). From the literature, however, it appears that *M. galloprovincialis* generally survives better than *M. edulis* possibly due to viability differences between the two species (Beaumont et al. 1993; Gardner et al. 1993). On the east coast of North America, Mallet and Carver (1995) found that among larger mussels, *M. trossulus* had a significantly higher survival than *M. edulis*, but there was no significant difference among smaller mussels. Based on the literature and

the data obtained in this study, it is difficult to make a definitive argument for which mussels (native or introgressed) have the potential for the fastest growth and highest survival on the west coast of North America. It is therefore hard to determine which species would be the best choice for aquaculture purposes on the west coast of B.C. Clearly more complete comparisons between the three mussel species in B.C. waters are warranted.

The limited number of introgressed mussels found in this study is the result of a decrease in introgressed mussels at Chemainus, B.C. (Chapter 1). Logistic difficulties with genotyping individuals also presented limitations in comparing the two groups. The genotyping of dead mussels was especially difficult, due to the lack of tissue upon collection, which made extraction of DNA for analysis problematic. Ideally, the mussels should have been screened prior to inclusion in this experiment for genotype; however, the effect of such sampling on survival and growth was, at that time, unknown. Chapter 3 describes a technique that could circumvent this problem in future studies. Mussels would be genotyped at the beginning of the experiment to allow selection of an equal proportion of both native and introgressed mussels to allow for more meaningful analysis (Yanick and Heath 2000).

2.5.2. Experiment 2: Transplanted vs Local Mussels.

The marked difference in performance between the transplanted and local mussels likely reflects local adaptation to environmental differences between the Chemainus and YIAL sites (Figures 2.4, 2.5). That is, the CH mussels may be adapted to local conditions at Chemainus, and hence did not perform optimally at YIAL. Local adaptation generally arises from selection pressures resulting from differing environmental conditions. Individuals that exhibit traits that enhance survival or reproductive success under these environmental conditions will be selected for, thus driving the divergence of populations (Hilbish and Koehn 1985; Rawson and Hilbish 1991; Taylor 1991; Boersma et al. 1999).

The most obvious environmental difference between the two sites, which may have contributed to the growth and survival differences in this study, was temperature. Although temperatures at the two sites were similar over the winter, summer and early fall temperatures were notably different (Figure 2.4a, 2.5a). These differences may explain how temperature might be a critical variable to which mussels must adapt. Salinity was unlikely a factor since mean salinity did not differ significantly ($F = 2.27$, $p \geq 0.171$) between the two sites (mean 26.55 ‰, S.E. 1.12, (YIAL), 24.82 ‰, S.E. 0.59 (CH)). Since there is no extreme water movement at either site, physical disturbance was also unlikely a factor. Nutrient loads were likely similar at the two sites, due to loading from the fish farm at YIAL and the boats at Chemainus. Although thermal adaptation appears to be a likely candidate for the differences in growth and survival in this study, in general, attributing physiological adaptation to any one environmental factor is questionable due to the complex interaction of such variables.

The cages used in this experiment (which allowed for the monitoring of individual mussels) were not likely to have had a negative effect on the growth or survival of the mussels, as each cage was designed to allow water and food to flow in and around the mussels. A study examining growth in *M. edulis* over 200 days (Page and Hubbard 1987) also found no difference in growth of caged and uncaged mussels. The cages may actually have fostered greater growth in the mussels, as Okamura (1986) found that isolated or small clumps of mussels had a greater growth rate than mussels in larger clumps.

Generally, growth in mussels (*Mytilus* spp.) has been found to be rapid during the spring and summer (temperatures 10 – 20 ºC) and slow or absent during the colder months (below 5 ºC) in temperate waters (Almada-Villela et al. 1982; Seed and Suchanek 1992; Blanchard and Feder 2000). In the present study, although both mussel groups displayed an increase in growth rate that coincided with an increase in water temperature, the spring months prior to this growth spurt were marked by an overall decrease in growth rate that

was not noted in the following spring. Overall, the growth differences between the transplanted and local mussels, although small and transient, were present. Although the transplanted (Chemainus) mussels remained larger than the local (YIAL) mussels throughout the experiment, they generally maintained a lower RGR. Larger mussels have a lower relative growth rate than smaller mussels because shell length is in the denominator. Relative growth represents the allocation of particular sized mussels to growth, and as such is biologically and aquaculturally relevant. In addition, other studies have shown that food availability and quality were the main determinants of growth rate in mussels (Ceccherelli and Rossi 1984; Page and Hubbard 1987; Frechette and Bourget 1987). It is possible that the higher summer temperatures at Chemainus provide a greater abundance of food and that food availability formed the basis of the environmental selection pressure. Speculation as to the mechanism of local adaptation is premature, but future studies could examine differences in food quality and quantity at the two sites to possibly clarify the cause of local adaptation.

Overall the transplanted mussels did not survive as well as the local mussels (Figure 2.5). A marked increase in mortality in both groups followed a 5-7 ºC increase in water temperature at YIAL (Figure 2.5a). The cause of this "outbreak" mortality may be attributed to increased metabolic stress related to spawning in the spring/summer. This may also be why RGR decreased in the spring/summer months. Increased metabolic stress in mussels has been correlated with high water temperatures and reduced food quality in August when mass mortality of mussels has been found to occur (Emmett et al. 1987; Tremblay et al 1998a and b). However, this has been shown to be more of an effect in larger mussels which expend more energy on reproductive output then smaller mussels (Worrall and Widdows 1984). Therefore, this is unlikely the cause of the "outbreak" mortality in this study. The increase in temperature may have been a factor, but increases in temperature are more of a detriment to intertidal mussels where they are exposed to high

temperature fluctuations and desiccation (Tsuchiya 1983). As the mussels in this study were reared subtidally, the increase in water temperature was not likely to have been the primary cause of the mortality. One possibility could be haemocytic neoplasia, the abnormal blood disorder that has been recorded in *Mytilus* mussels during the late summer and early fall in British Columbia (Bower 1989). This disorder may be the cause of the early mortality syndrome of west coast *M. trossulus* mussels in the summer-fall of their second year when mussels are approximately 4 cm (Bower 1989). Studies determined that the environment may have some influence on the development of haemocytic neoplasia and survival, although there was no correlation between the incidence of haemocytic neoplasia and mortality in mussels (Bower 1989). In a study of the Pacific oyster (*Crassostrea gigas*), however, increases in water temperature and elevated nutrient levels were suggested to act as external stressors which made oysters more susceptible to pathogens (Friedman et al. 1991). The increase in temperature noted in this study may then have made the mussels more susceptible to a potential pathogen. This mortality of native mussels in B.C. is the reason aquaculturists want to import alien mussels for commercial purposes.

An alternative possibility may be that the differences in performance were a result of increased stress to the Chemainus mussels due to the longer transport and handling process. This, however, is not likely the primary cause of the difference in performance between the YIAL and CH mussels. Had the transport stress differential been the cause of performance differences, a more immediate effect would have been observed (i.e., soon after transfer). Since significant differences were not noted until later in the experiment, and performance differed in each cage, handling was not considered to be the cause of performance differences between the two groups.

As this study was originally intended to test for growth and survival differences between native and introgressed mussels, it was not designed specifically to examine local adaptation. Based on the evidence from this study suggesting the presence of substantial

local adaptation in mussels in Georgia Strait, additional experiments could be designed to specifically examine local adaptation and clarify the environmental cause(s) of local adaptation. For example, the use of a reciprocal transplant design would allow for a more thorough examination of the differences in growth and survival between the two *M. trossulus* populations. In addition, multiple environmental factors could be monitored to determine whether the local adaptation was the result of a combination of effects or whether local adaptation was the result of a single dominant environmental variable.

The results of this study demonstrate the existence of local adaptation in B.C. blue mussels and the potential importance that it may have for shellfish aquaculture. Few studies exist that have shown local adaptation, thus, it is necessary to gain more information to enable the productive culturing of mussels in Georgia, Strait, B.C. Broodstock selection methods need to be reconsidered, since this study shows that significant variation can exist in commercially important variables, such as growth and survival, among sites. Further work needs to be done to compare the performance of local (*M. trossulus*), imported (*M. edulis*, *M. galloprovincialis*) and introgressed mussels in B.C. waters.

Chapter 3: A Nondestructive Technique for the Recovery of DNA from Bivalves (published in part in Yanick and Heath 2000).

3.1. Abstract:

The increasing use of molecular genetic techniques for ecological and evolutionary applications has led to the need for non-destructive methods of obtaining DNA. This study resulted from the logistic difficulties with the methodology in Chapter 2 that was to be a comparison of survival and growth rates between native and introgressed mussels in Georgia Strait. The technique described in this paper would enable the genotyping of individuals prior to their selection that would allow for the inclusion of an equal proportion of both native and introgressed mussels. In this study, 50 blue mussels (*Mytilus* spp.) were collected in each of three size classes: small (10-20 mm), medium (20-30 mm) and large (30+ mm). In each size class, hemolymph was extracted from 25 mussels while the remaining 25 mussels were kept as controls. All groups were monitored for 384 days, during which time no significant differences in growth or survival were found. DNA was extracted from the hemolymph and was polymerase chain-reaction (PCR) amplified with ITS and Glu-5' species-specific markers. Mussels were genotyped using these markers successfully from 81% and 92% of the samples respectively, and all were determined to be *Mytilus trossulus* (Lamark). This non-destructive technique of hemolymph extraction for DNA analysis enables molecular investigations of rare or limited populations or species, and for life history studies were survival of the organism is required. The technique may also have application for further distribution and abundance surveys (Chapter 1) as it would enable the sampling of sites with limited population size because mussels would not have to be removed and killed for genotypic analysis.

3.2. Introduction:

Molecular techniques used to genetically characterize individuals have become increasingly common in the study of the ecology and evolution of marine invertebrates (Mitton 1994). Prior to the use of molecular techniques to study genetic variation, biologists relied on morphological and behavioural differences to study population variation (May 1998). The first molecular technique in widespread use was allozyme electrophoresis. Allozyme studies have been performed on many species of marine bivalves to distinguish sympatric species (McDonald et al 1991; Day et al. 2000), determine distributions and population structure (Penney and Hart 1999; Suchanek et al. 1997; Sarver and Foltz 1993; McDonald et al. 1991; McDonald and Koehn 1988), map hybrid zones and introgression (Gardner 1995;), and to determine ecological microhabitat structure (Bowen 1982; Freeman-Gallant 1996; Scribner et al. 1997). Since then, molecular techniques have been developed which detect variation in DNA sequence directly, and these techniques have revealed additional genetic variation providing other possible choices of molecular genetic markers (Mitton 1994). In comparison to allozyme analysis, which requires large amounts of fresh tissue for analysis, the new techniques require very little sample DNA making them useful for studies of populations of rare organisms, or those requiring the survival of the study organism.

Polymerase chain reaction (PCR) is a particularly important tool in the study of molecular ecology and population genetics. This technique enables researchers to produce large quantities of specific DNA segments from as little as a single template molecule (Hoelzel and Green 1998). As little DNA is required initially, PCR makes it possible to perform DNA studies when the quantity and/or quality of DNA is limited (Hoelzel and Green 1998), such as with dried or preserved samples, or for nondestructive sampling (Mitton 1994). Advances in the use of PCR have made it possible to redefine species and populations based on their genetic structure (Lynch and Milligan 1994). PCR techniques

have been used in many genetic structure studies involving plants (eg., Williams and Fiori 1996; Stewart and Excoffier 1996), insects (eg., Ross et al. 1997), plant-insect populations (eg., Michalakis et al. 1993), fish (eg., McGauley and Mulligan 1995), and aquatic invertebrates (eg., Viard et al. 1997; David et al. 1997; Heath et al. 1995 and Sarver and Foltz 1993). In the *Mytilus* species complex, PCR-based species markers have been used for conservation, ecological, and evolutionary applications (Heath et al. 1995; 1996; Rawson et al. 1996). The work done by Heath et al. (1995) and Rawson et al. (1996) led to the creation of PCR-based genetic species markers that discriminated the *Mytilus* sibling species and have helped clarify the genetic structure of this complex of sibling species. Genetic characterization has also clarified the population genetic structure in a variety of other bivalves such as scallops (eg., Herbinger et al. 1998; Manuel et al. 1996), clams (eg. David et al. 1997) and oysters (eg., Ignacio et al. 2000; Allen and Gaffney 1993), as well as aiding in investigations into the ecology and life history of bivalves with planktonic phases (Toro 1998).

Typically, shellfish are destructively sampled in order to sample tissue for DNA extraction (Taberlet et al. 1999). Emphasis is now being placed on using sampling techniques that do not compromise the health and survival of the organism. For example, nondestructive sampling generally involves capturing the target organism, taking an invasive sample without killing it and then releasing it, while noninvasive sampling involves the sampling of DNA from sources left behind by the target organism without catching or disrupting it (Taberlet et al. 1999). Although destructive sampling has been used in many shellfish studies (Toro 1998; Herbinger et al. 1998; Suchanek et al. 1997; Hare et al. 1996; Heath et al. 1996; Heath et al. 1995; Sarver and Foltz 1993), this method is not an option for investigations into growth or survival, studies of small populations or rare organisms, or examination of individuals or populations for use as broodstock in aquaculture. These studies, which rely on the survival and fitness of the target organism, require a technique

that allows the collection of DNA without harming the organism. It is important that any potential technique be tested for even minor adverse effects on the survival or growth of the target organism. These parameters are important to test when developing new techniques because even small impacts on growth or survival could bias any observations of performance, or possibly jeopardize the survival of valuable broodstock.

This study describes a non-destructive sampling technique for collecting hemolymph for DNA extraction from mussels and was the result of a need for a non-destructive method for sampling DNA from mussels for the purpose of monitoring survival and growth rates (Chapter 2). Hemolymph, which acts as blood in bivalves, is made up of mostly water, but does contain cells, including nucleated hemocytes (Morse and Zardus 1997) and is, in part, responsible for the transportation of digestion products throughout the body (Brusca and Brusca 1990). Specifically, hemolymph was sampled from three size categories of blue mussels (*Mytilus* spp.), DNA was extracted, and fragments were amplified using PCR with species-specific markers. The survival and growth of both the hemolymph-extracted and control mussel groups were followed for over one year to ascertain whether this technique resulted in decreased survival and/or growth.

3.3. Materials and Methods:

3.3.1. Field Sampling:

Mussels were collected subtidally from the docks at Yellow Island Aquaculture Ltd. located on Quadra Island, British Columbia, Canada. The length (from tip of the beak to the posterior edge) of each mussel shell was measured with calipers to the nearest 0.1 mm and sorted into small (10 - 20 mm), medium (20 – 30 mm) and large (30 + mm) size categories each containing 50 mussels (Figure 3.1). In each class, hemolymph was extracted (50 µl – 200 µl) from 25 mussels, while the other twenty-five mussels (control) were handled, but

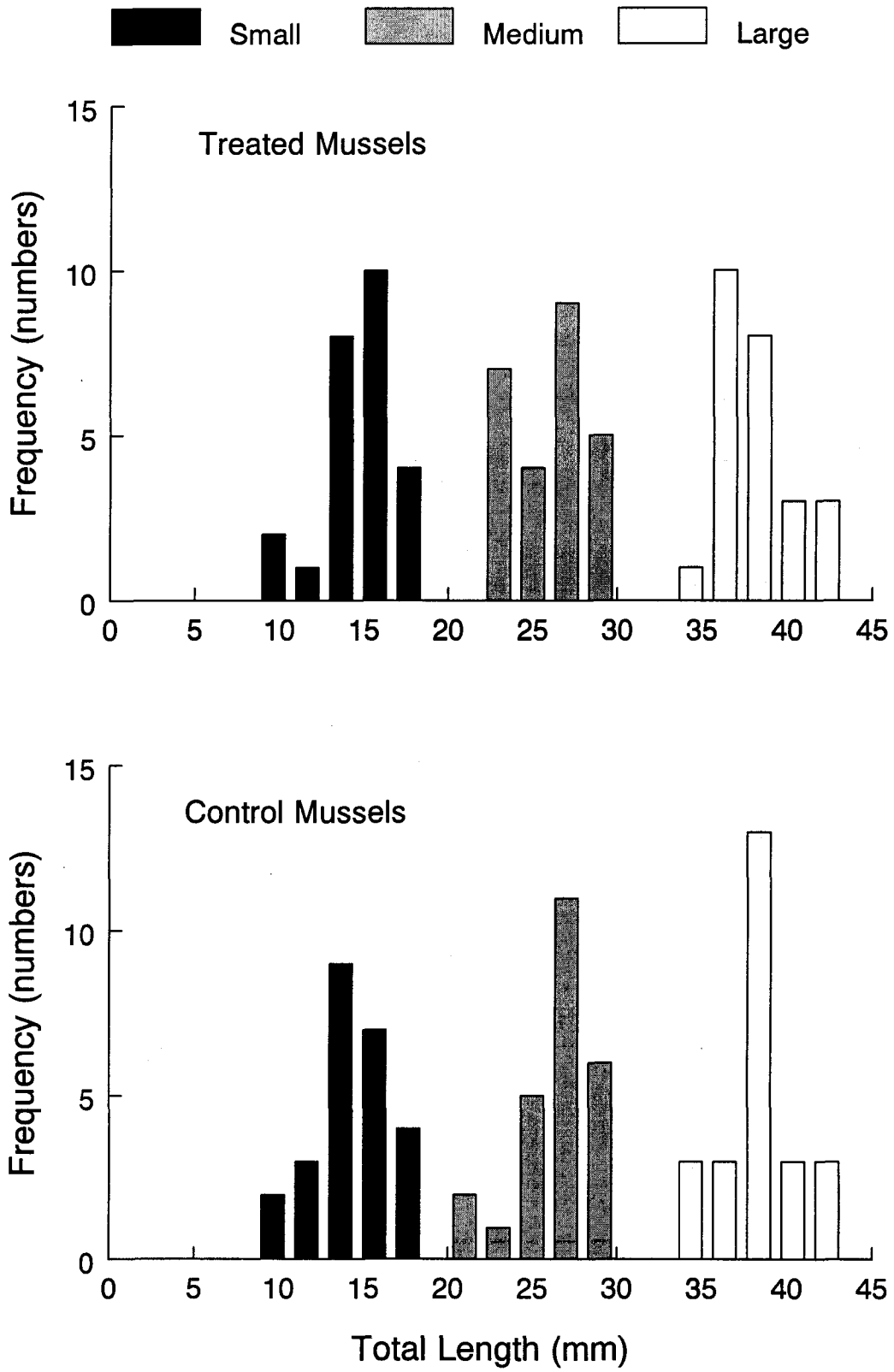


Figure 3.1. Size distribution of mussels (control and treated) collected at Yellow Island Aquaculture Ltd. on April 3, 1998. Small mussels (10 – 20 mm), medium (20 – 30 mm), large (30+ mm).

not sampled. A 1-cc syringe (22 gauge, 1.5-inch needle) was inserted through the rear hinge joint and hemolymph was extracted. The extracted hemolymph was expelled into 1.0 ml of 95% ethanol and stored at room temperature. After sampling, mussels were placed in six cages: 3 containing the hemolymph-extracted mussels (one of each size class) and 3 containing the control (one of each size class). The cages, which were 5cmX5cmX10cm and were slotted to enable free water flow, were then hung approximately 1 m below the surface at the original collection site.

3.3.2. DNA Analysis:

The hemolymph and alcohol were centrifuged (13000 rpm, 15 min), the liquid removed, and the pellet dried (LABCONCO Centrivap Concentrator) at 60°C for 8 minutes. Mussel DNA was extracted from the hemolymph and genotyped in the same manner as Chapter 1. The dried cells were digested overnight in 200 µl of lysis buffer (10mM Tris HCl pH 8.0, 15 mM EthyleneDiamine Tetra Acetate, 0.5% Sodium Dodecyl Sulphate) and 125 µg of proteinase K while rocking at 37°C. The solution was then extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1), followed by isopropanol precipitation (Heath et al. 1995). The extracted DNA was resuspended in 100 µl of double-distilled water and then PCR amplified following the ITS protocol described in Heath et al. (1995) and the Glu-5' protocol described in Rawson et al. (1996). The PCR products were visualized on a 1.8% agarose gel stained with ethidium bromide (Figure 3.2). Individual mussels were scored for genotype at each marker locus on the basis of a diagnostic RFLP (ITS; Heath et al. 1995) or using an automated DNA sequencer to determine amplified fragment length (Glu-5'; Rawson et al. 1996).

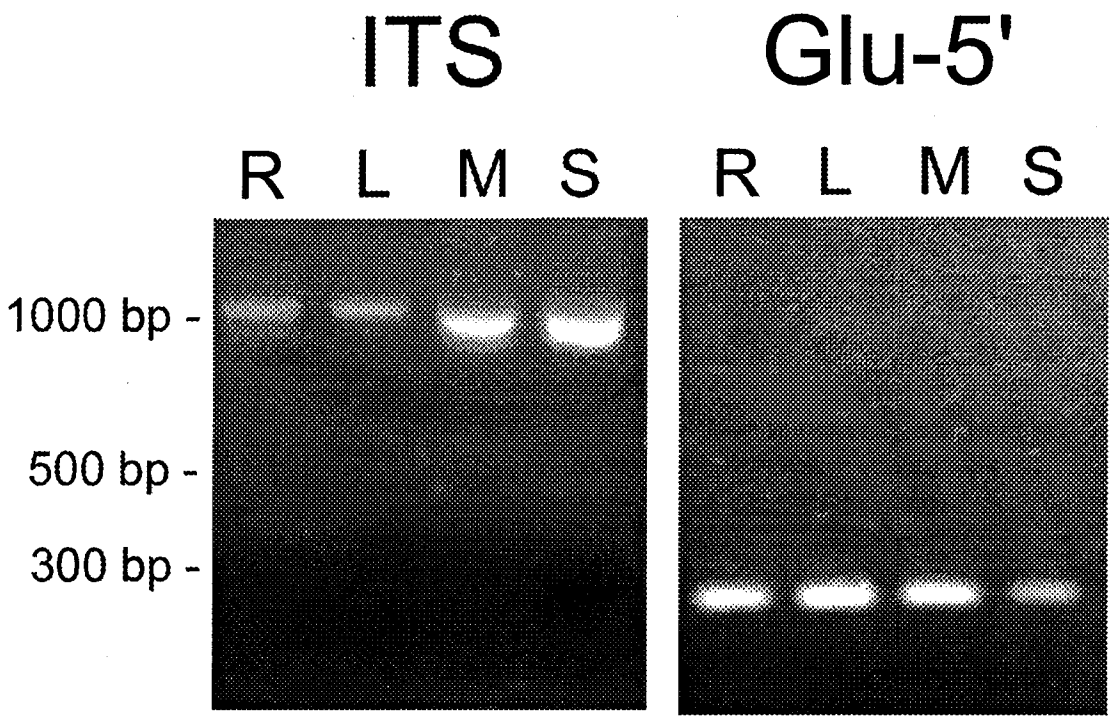


Figure 3.2. Agarose gel electrophoresis picture of PCR-amplified DNA fragments (size in base pairs, bp) using the ITS and Glu-5' species specific markers. The lanes are PCR results using DNA from a destructive extraction method (R) and DNA extracted from hemolymph taken non-destructively from mussels in three size classes (L, large; M, medium; S, small).

3.3.3. Growth and Survival:

At three sampling times from April 3, 1998 to April 22, 1999 (58, 140 and 384 days), the mussels in this experiment were measured and survivors counted. Student's t-test (length) and chi-square tests (survival) were used to determine whether differences existed both between the experimental and control groups at each size class as well as across the three size classes at day 58 and day 384. Day 58 comparisons were made to test for short-term effects, while day 384 comparisons were made for long-term effects.

3.4. Results:

At the beginning of the testing period (April 3, 1998), the control mussels in the small category had a mean total length of 15.64 ± 0.1 mm while the hemolymph-extracted mussels had a mean total length of 15.87 ± 0.1 mm. In the medium category the mean total length of the control mussels was 26.81 ± 0.1 mm and 25.96 ± 0.1 mm in the treated group (Figure 3.1). In the large size class, the mean total length of the hemolymph-extracted group was 37.08 ± 0.1 mm while the mean total length of the control group was 36.91 ± 0.1 mm (Figure 3.1).

DNA was successfully PCR-amplified from 61 of the 75 samples (81%) for ITS, and from 69 of the 75 samples (92%) for GLU (Figure 3.2). All mussels were determined to be *Mytilus trossulus*.

The hemolymph extraction technique was found to have little effect on either survival (Figure 3.3) or growth (Figure 3.4). At day 58 and day 384, the survivorship of the hemolymph-extracted mussels was not significantly different than the survivorship of the control mussels in any of the size categories ($P>0.05$). At day 58, the control mussels were slightly larger than the hemolymph-extracted mussels in the small size category ($t= -2.448$, $P= 0.018$), but there was no significant differences in either the large or the medium size

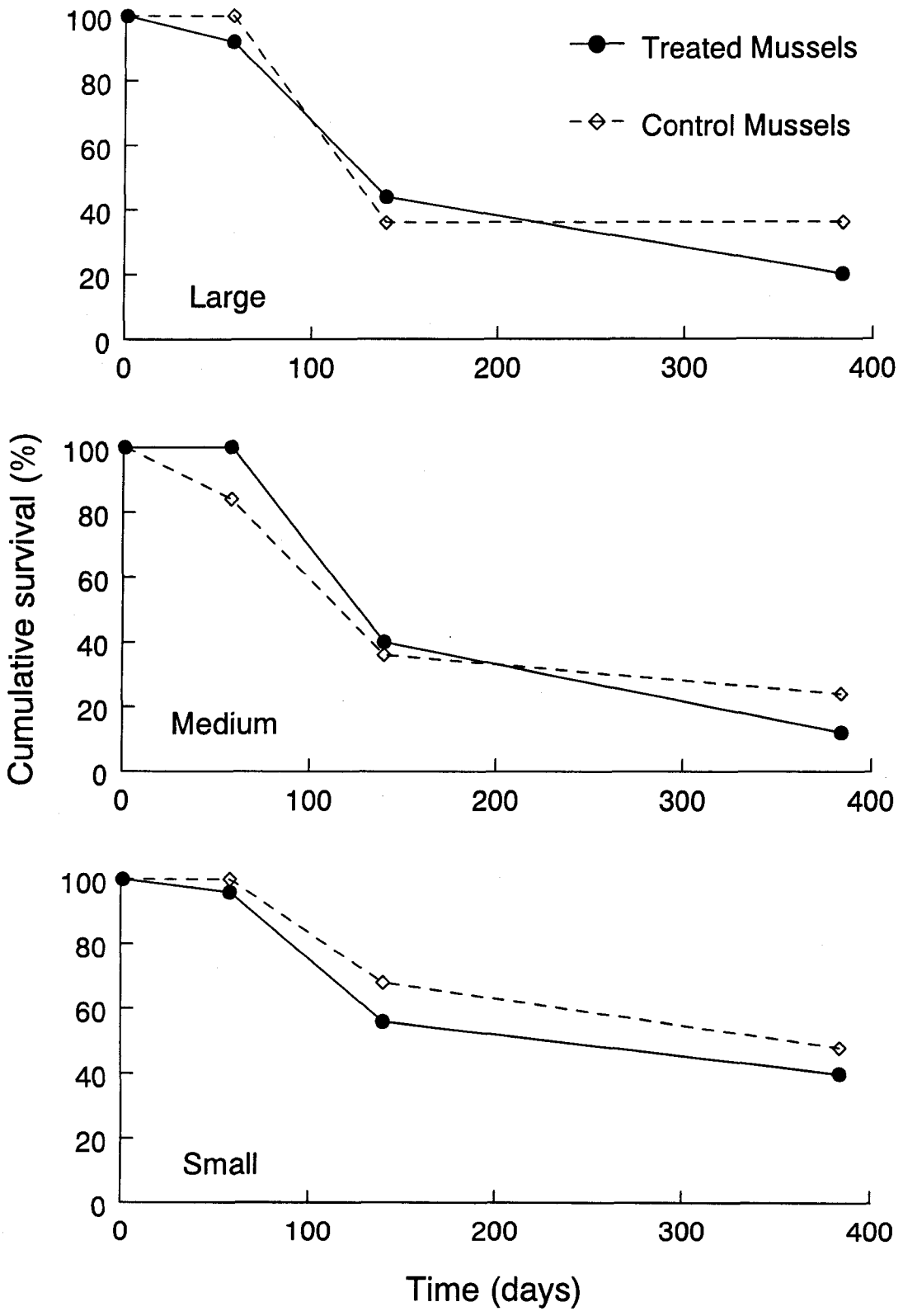


Figure 3.3. Comparison of cumulative survival of the three mussel size classes between April 3, 1998 and April 28, 1999 for both the control and treated mussel groups. The closed circles with solid lines represent the treated (hemolymph extracted) mussels while the open diamonds with dashed lines represent the control mussels.

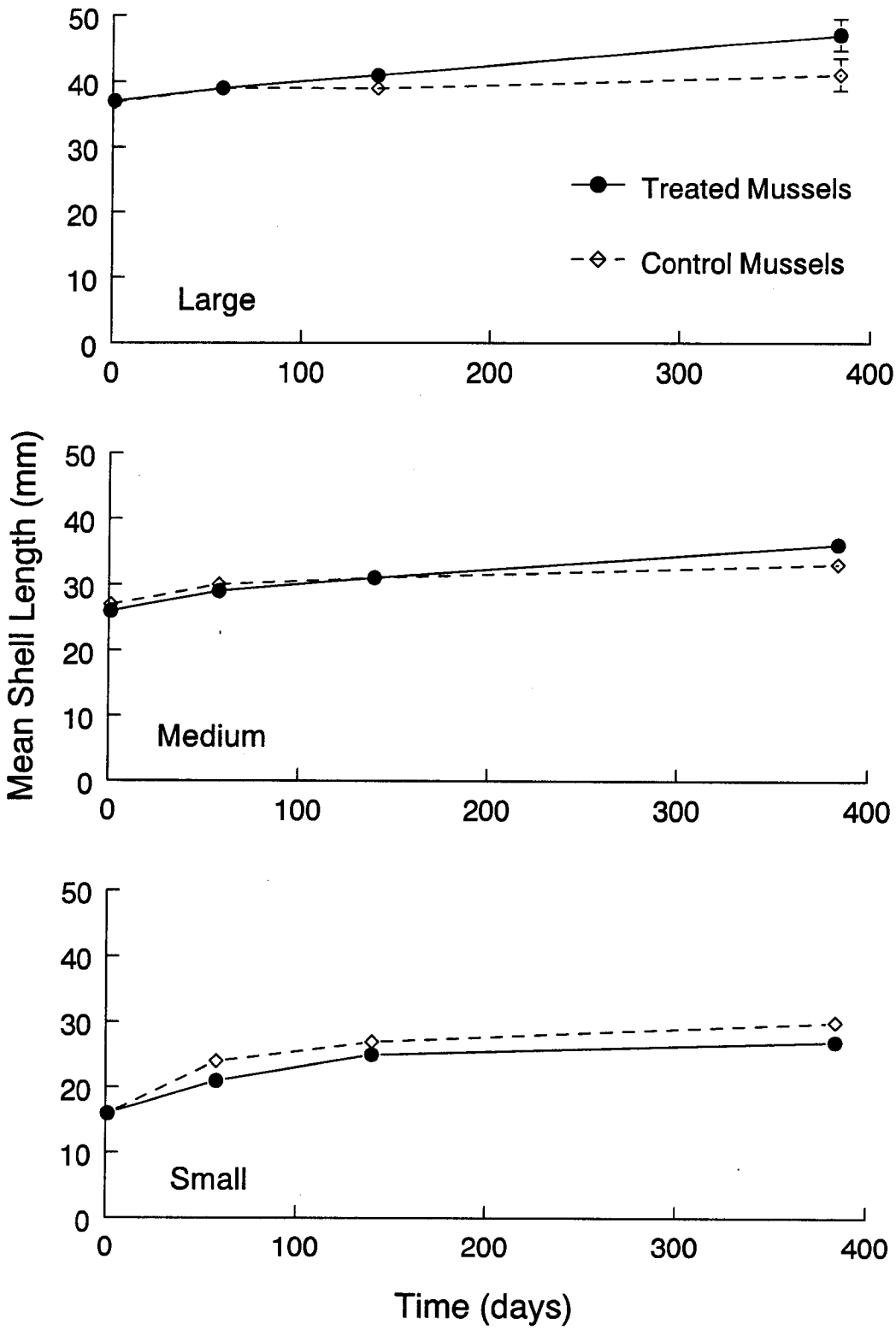


Figure 3.4. Comparison of mean shell length (mm) of the three mussel size classes between April 3, 1998 and April 28, 1999 for both the control and treated mussel groups. The closed circles with solid lines represent the treated (hemolymph extracted) mussels while the open diamonds with dashed lines represent the control mussels.

categories ($p>0.50$). At day 384, the hemolymph-extracted mussels were larger than the control mussels in the large size category ($t= 2.412$, $p= 0.033$), but there was no significant difference in the medium or small size categories ($p>0.10$) (Figure 3.4). In comparison across the size classes (small, medium and large), no consistent differences were found in survival or growth. The smallest mussels sampled (10 – 20 mm) had similar survival and growth to the larger size categories (20 – 40+ mm).

3.5. Discussion:

A wide range of mussel sizes was sampled using a non-destructive method of DNA sampling, and DNA was successfully extracted for PCR purposes from most of the mussels, including those in the small category. The success rate of this study was comparable to that of Heath et al. (1995) who used destructive tissue sampling methods (also Chapter 1 and 2). The technique described here is a useful tool for fieldwork, as it does not require the killing of the organism under study. Furthermore, we found no consistent effect of hemolymph sampling on either survival or growth of the mussels. Although this is not surprising for the larger mussels, it is unexpected for the small animals, as the extraction of a large portion of the organism's body fluid would be expected to negatively affect the organism's growth and/or survival. This ability to nondestructively sample smaller animals will be especially useful to aquaculturists for the determination of potential broodstock.

Although mussels were used in this study, this technique is applicable to other bivalve species. For example, Moore et al. (1991) used a similar technique to extract hemolymph from mussels to study systemic neoplasia, although after extraction, the mussels were dissected for further analysis. Manuel et al. (1996) used a similar technique on scallops (*Placopecten magellanicus*); however, they did not test for potential growth or

survival effects of their sampling method. Marsh et al. (1995) presented a "nondestructive" methodology using hemolymph for assessing *Perkinsus marinus* infections in eastern oysters. Once the hemolymph was extracted, however, the study oysters were later processed, which does not coincide with Taberlet et al.'s (1999) definition of nondestructive sampling. With more studies relying on the health and survival of the study organism, it is important that the techniques employed do not impact the survival of the organism or its general health, as negative impacts could bias observations of fitness or endanger future broodstock.

The technique will be useful for studies of the ecology and population dynamics of bivalves where destructive sampling of the organism is either not permitted, or not desirable for the experimental design. As no significant effect on survival or growth was found from this study, this technique will enable researchers to identify and examine populations without threatening the population itself. This technique will also have wide-reaching applications in the aquaculture industry where genetic characterization of potential broodstock is required and can be used to ensure uniformity of species within the farm population.

Unfortunately this technique was not used in the survival and growth examination described in Chapter 2 because the results of this nondestructive technique were not determined until the end of the survival and growth period of the study in Chapter 2. If the survival and growth comparison study was to be repeated, using this nondestructive technique would enable the determination of species at the beginning of the time period instead of waiting until the end. The study population could then be selected for a representative portion of all species of interest rather than assuming the sample contained all species.

General Conclusion

The primary purpose of the research in this thesis was to examine the genetic and ecological implications of mussel introductions to Georgia Strait, British Columbia. The study was motivated by ongoing suspected introductions via ballast water discharge and by aquaculture escapees. The potential impact of those introductions was not understood, nor was the status of the invasion known. The secondary goal of this work was to examine the potential for ecologically sound mussel aquaculture in BC. Specifically, the thesis consists of 3 projects designed to 1) examine the invasion dynamics of blue mussels (*Mytilus* spp.) at both large and small spatial and temporal scales in Georgia Strait, British Columbia, 2) compare, for aquaculture purposes, the survival and growth rates of local and transplanted mussels (*Mytilus* spp.) in Georgia Strait, and 3) develop a non-destructive technique for the recovery of DNA from bivalves.

The examination of spatial and temporal variation within the *Mytilus* species hybrid zone in Georgia Strait indicated that the distribution and abundance of the alien mussels were not expanding and that the introgression of alien alleles into the native mussels did not appear to be accelerating at either the large or small scale. It was determined that a *Mytilus* hybrid zone exists within Georgia Strait, BC which resembles a mosaic type of hybrid zone but is unstable and appears to be maintained by introductions (both human introductions and migrations). This novel "immigration mosaic hybrid zone" is of interest for conservation, ecological, and evolutionary theory because it appears to be a newly forming hybrid zone that can be examined for selection effects, gene flow, and immigration and mating effects. Continued sampling of this area would provide further insight into whether the processes involved are resulting in successful or unsuccessful invasions and further hybrid zone development. Generally, scientific study has focused on successful invasions and little examination has been done on non-successful invasions. The Georgia Strait hybrid zone, with its on-going unsuccessful invasion, provides an opportunity to study the

dynamics of non-success. Given that introductions and/or invasions are likely to continue in this area, however, it is probable that eventually the proper environmental conditions will exist to enable a successful invasion. The dynamics of this hybrid zone will also likely change with the increase of mussel aquaculture in the area. This is the reality of what Carlton and Geller (1993) termed "ecological roulette." The continuation of introductions of species into new areas will sometimes be harmless, although it can sometimes cause irreversible damage in an area.

The examination of survival and growth differences of the *Mytilus* blue mussels, both between native and introgressed species as well as between different populations of native species in Georgia Strait, was conducted to determine the commercial value of the native mussels (*M. trossulus*). Due to small numbers of introgressed mussels, a definitive determination of survival/growth differences between the introgressed and native mussels could not be made; however, as a result of the limitations of the methodology used in this study, a technique was developed where mussels could be genotyped prior to the inclusion in growth/survival studies, thus allowing pre-screening for larger sample sizes of alien mussels (Yanick and Heath 2000). The described technique will likely represent a valuable methodological tool for bivalve ecological, evolutionary, and physiological studies. Interestingly, evidence suggests an unexpected presence of significant local adaptation effects in the native mussels in Georgia Strait. This could prove to be problematic for the mussel aquaculture industry as the transfer of broodstock between environmentally differentiated sites could result in a loss of performance if the imported mussels are not adapted to the new conditions. The results of this study further demonstrate the absence of panmictic population structure in these mussels, despite their exceptional dispersal potential.

The demand for mussel aquaculture in Georgia Strait is growing and at this point, the Department of Fisheries and Oceans has already permitted the importation of *M. edulis*

and *M. galloprovincialis* for aquaculture purposes to various areas in Georgia Strait (pers. com. D. Kieser, Fish Pathologist, Fisheries and Oceans Canada). However, despite the desire to import commercial species used elsewhere in the area, it is necessary to further evaluate the commercial potential of the native species, *Mytilus trossulus* and existing introgressed mussels. Further study into the causes of the early mortality of *M. trossulus* may lead to solutions that would enable a larger percentage of these mussels to reach harvestable size. It is also necessary to determine whether the transfer of these mussels to different sites within Georgia Strait would be detrimental to the commercial industry. Furthermore, it is imperative that further study of the developing hybrid zone in Georgia Strait be conducted. Not only does hybridization have consequences for the commercial industry, through potential reduced fitness of all species in the area, it also has potential consequences for the genetic make-up and ecology of the species themselves.

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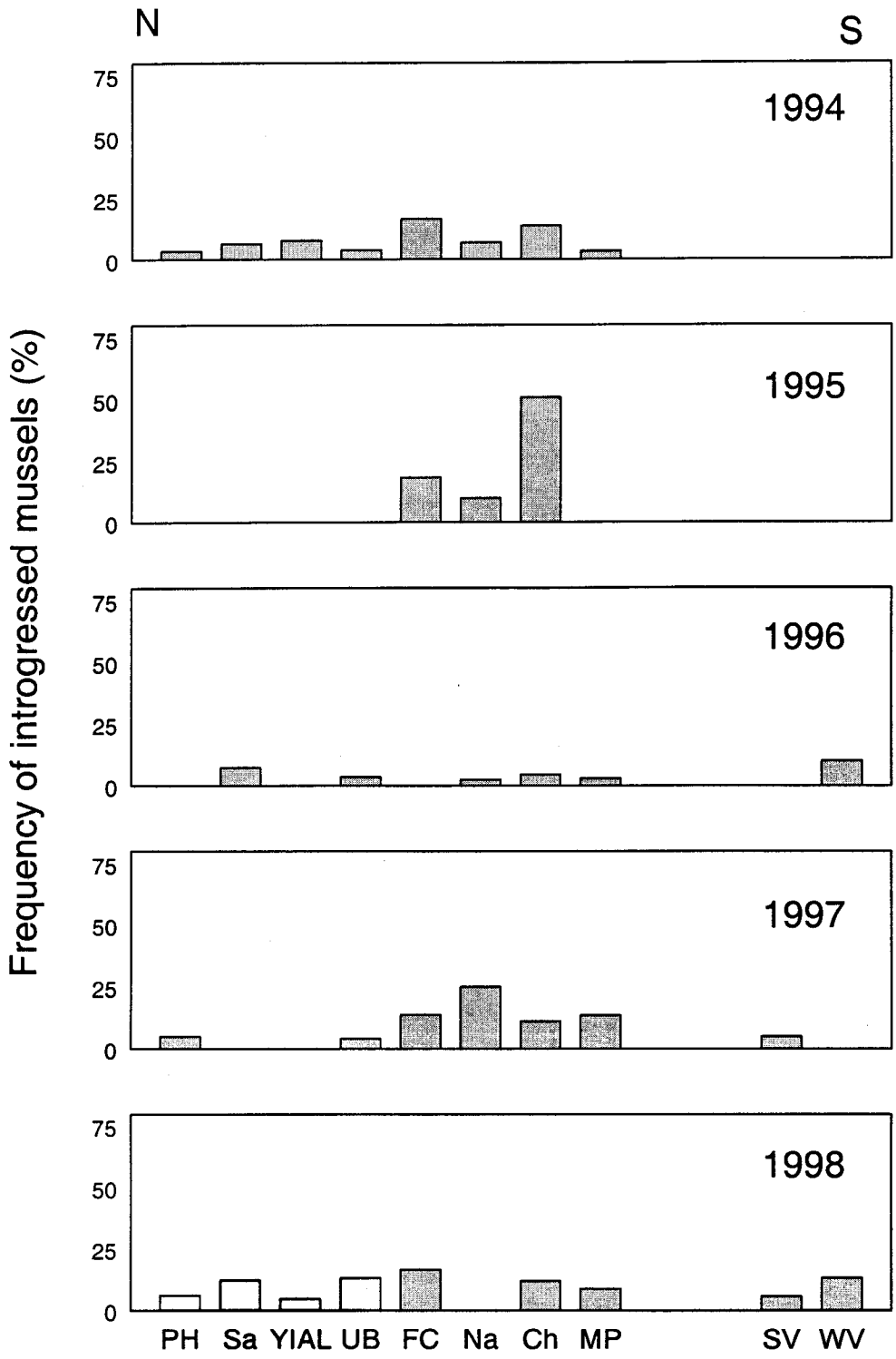
Appendices

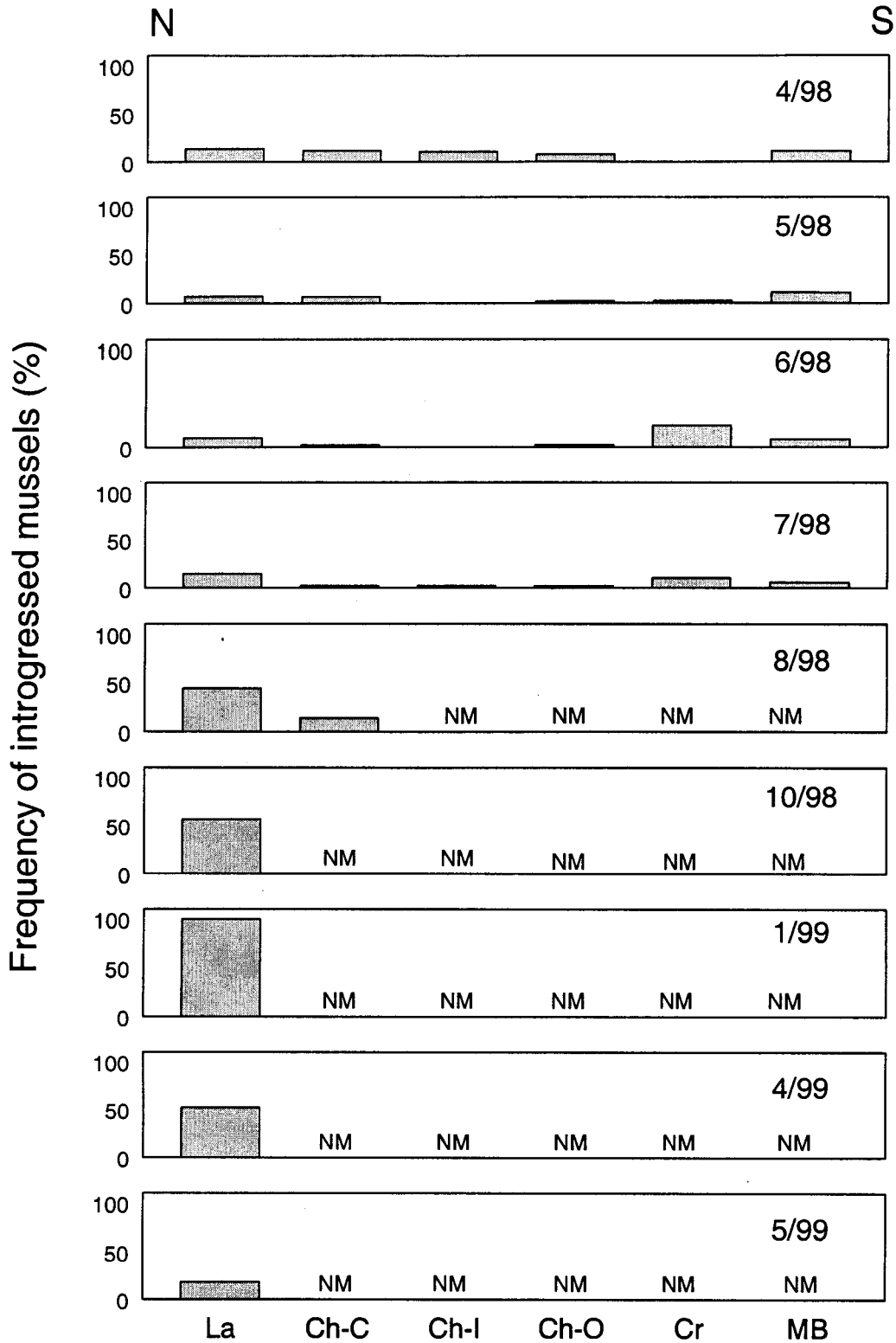
Appendix I. Spatial and temporal distribution of introgressed mussels in Georgia Strait, B.C. from north (Port Hardy) to south (West Vancouver) between 1994 and 1998.

Appendix II. Spatial and temporal distribution of introgressed mussels in Georgia Strait, B.C. from six sites within and surrounding Chemainus, B.C. from north (Ladysmith) to south (Maple Bay) between April, 1998 and May, 1999.

Appendix III. Schedule for the sampling of mussel growth and survival between March 20, 1998 and July 15, 1999.



Appendix I. Spatial and temporal distribution of introgressed mussels in Georgia Strait, B.C. from north (Port Hardy) to south (West Vancouver) between 1994 and 1998. PH – Port Hardy, Sa – Sayward, YIAL – Yellow Island Aquaculture Ltd., Quadra, UB – Union Bay, FC – French Creek, Na – Nanaimo, Ch – Chemainus, MP – Moses Point, SV – South Vancouver (Kitsilano), WV – West Vancouver (Horseshoe Bay).



Appendix II. Spatial and temporal distribution of introgressed mussels in Georgia Strait, B.C. from six sites within and surrounding Chemainus, B.C. from north (Ladysmith) to south (Maple Bay) between April, 1998 and May, 1999. La – Ladysmith, Ch-C – Chemainus, commercial dock, Ch-I – Chemainus, inner dock, Ch-O – Chemainus, outer dock, Cr – Crofton, MB – Maple Bay. "NM" designates no mussels found.

Appendix III

Schedule for the sampling of mussel growth and survival between March 20, 1998 and July 15, 1999. 'C' denotes collection. 'M' denotes mortality check. 'G' denotes specimen measurement. YIAL (locals) are the mussels collected from the fish farm on Quadra Island. CH-1, CH-2, CH-3 (transplants) are the mussels collected from Chemainus, B.C. on successive weeks (March 20, 1998, March 28, 1998, April 4, 1998).

Date	Days since start of trial	YIAL	CH-1	CH-2	CH-3
March 20, 1998	0	C	C		
March 28, 1998	8			C	
April 4, 1998	15	M	M	M	C
April 18, 1998	29	G/M	G/M	G/M	G/M
April 25, 1998	36	M	M	M	M
May 3, 1998	44	M	M	M	M
May 9, 1998	50	G/M	G/M	G/M	G/M
May 19, 1998	60	M	M	M	M
May 24, 1998	65	M	M	M	M
May 30, 1998	71	G/M	G/M	G/M	G/M
June 11, 1998	83	M	M	M	M
June 20, 1998	92	G/M	G/M	G/M	G/M
July 11, 1998	113	G/M	G/M	G/M	G/M
July 31, 1998	133	G/M	G/M	G/M	G/M
August 21, 1998	154	G/M	G/M	G/M	G/M
September 4, 1998	168	M	M	M	M
September 18, 1998	182	M	M	M	M
October 2, 1998	196	M	M	M	M
October 30, 1998	224	G/M	G/M	G/M	G/M
December 25, 1998	280	M	M	M	M
January 17, 1999	303	G	G	G	G
February 25, 1999	342	G/M	G/M	G/M	G/M
April 22, 1999	398	G/M	G/M	G/M	G/M
May 28, 1999	434	G/M	G/M	G/M	G/M
July 15, 1999	482	G/M	G/M	G/M	G/M